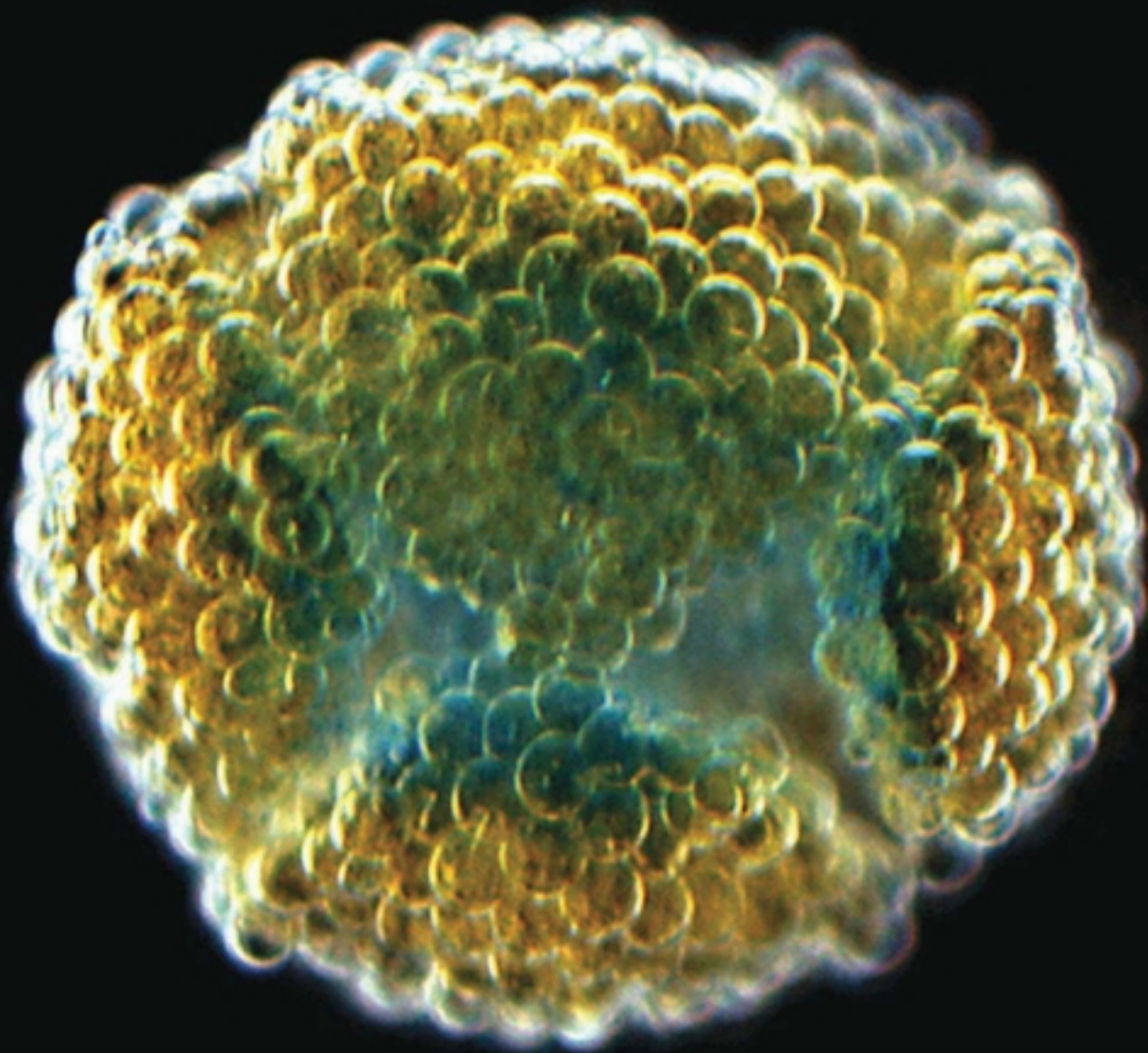


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# Science



 AAAS

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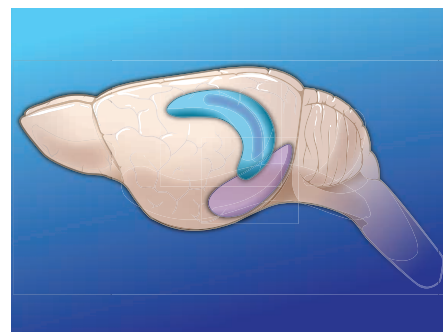
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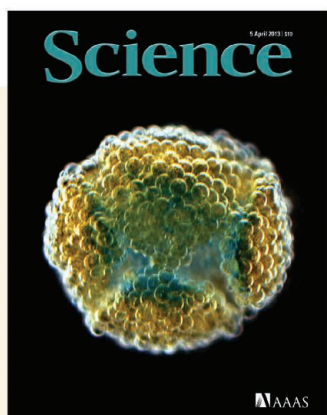
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### >> Science Podcast

Listen to stories on de-extinction, decoding dreams, Earth's inner movements, and more.

### >> Find More Online

Check out *Science Express*, our podcast, videos, daily news, our research journals, and *Science Careers* at [www.sciencemag.org](http://www.sciencemag.org).



## COVER

A network of picoliter aqueous droplets generated by 3D printing. After printing, the droplet network folded into a hollow sphere (diameter: 0.4 millimeters). In these designed tissue-like materials, adjacent compartments are separated by lipid bilayers and can communicate with each other and the environment. Such printed materials might be used to deliver drugs or, in the long term, to augment failing organs. See page 48.

Photo: Gabriel Villar

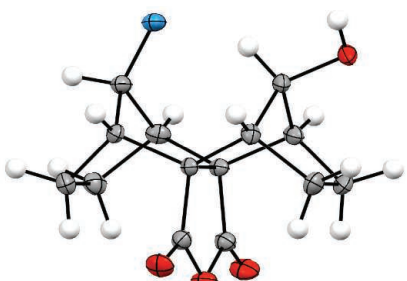
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## Reef Repair

Coral reefs suffer mass mortality because of coral bleaching, disease, and tropical storms, but we know much more about when, where, and how rapidly these ecosystems have collapsed than we do about their recovery. **Gilmour *et al.*** (p. 69; see the Perspective by **Polidoro and Carpenter**) studied a highly isolated coral reef before and after a climate-induced mass mortality event that killed 70 to 90% of the reef corals. The initial recovery of coral cover involved growth and survival of remnant colonies, which was followed by increases in larval recruitment. Thus, in the absence of chronic disturbance, even isolated reefs can recover from catastrophic disturbance.

## Bio-Inspired Synthetic Network

Collective behavior comes through the ability of neighboring objects to communicate and interact with each other. **Villar *et al.*** (p. 48; see the cover) produced three-dimensionally patterned, interconnected networks of lipid-bounded structures functionalized with transmembrane proteins, which allowed electrical communication along specific pathways.

## Cycling in Unison

Many small mammals, especially voles, display semi-regular cycles of population boom and bust. Given the fundamental importance of small mammals as basal consumers and prey, such cycles can have cascading effects in trophic food webs. **Cornulier *et al.*** (p. 63) collated raw data from vole populations across Europe collected over the past 18 years. Reduction in winter growth rate was common across a wide variety of habitats with very different local climates, suggesting the presence of a continental-scale climatic driver of vole populations.

## Thwarting HIV

Multiple genome-wide association studies have revealed that human leukocyte antigen (HLA) genes of the major histocompatibility complex locus have the strongest impact on HIV. In particular,

a single-nucleotide polymorphism 35 base pairs upstream of HLA-C shows significant association with viral load and protection against HIV. How HLA-C mediates these effects is unknown. **Apps *et al.*** (p. 87) now demonstrate that increasing surface expression of HLA-C is associated with reduced viral load and reduced rate of progression to low CD4<sup>+</sup> T cell counts in African and European Americans. High HLA-C expression likely promoted improved HIV control through a more effective cytotoxic CD8<sup>+</sup> T cell response. In contrast to HIV infection, high HLA-C expression was associated with a higher risk of the inflammatory bowel disease, Crohn's disease.

## How to Make Decisions

Recently, a number of methods to probe internal properties of nonlinear neural systems have been developed. In these methods, highly variable stimuli are used to explore the input space of the system. Neural responses are then studied using models that take advantage of the known trial-by-trial stimulus information. **Brunton *et al.*** (p. 95) adapted this combined approach to decision-making. Both in rats and humans, the diffusion constant of the drift-diffusion model of decision-making was zero, implying that the noise is all in the processing of sensory input and not in the evidence accumulator. In addition, rats gradually accumulated evidence for decision-making, with strong effects of sensory adaptation on gradual accumulation of evidence.

## A Light in the Dark

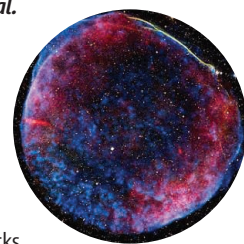
Carotenoids enhance the efficiency of photosynthesis by absorbing light at wavelengths inaccessible to chlorophyll. However, the mechanism underlying transfer of the absorbed energy from these pigments to the site of chemical catalysis is unclear. Studies have debated the involvement of a "dark" state in the carotenoid electronic structure—a state inaccessible by direct absorption populated by partial relaxation of the initial excited state. **Ostroumov *et al.*** (p. 52) used two-dimensional electronic spectroscopy to provide evidence of this state.

## Fluorine Learns to Share

Though halides typically coordinate to just one carbon center, their transient coordination to a second carbon (forming a positively charged bridge) explains the spatial dynamics of many reactions. However, unlike chlorine, bromine, and iodine—which can all form such halonium ions—fluorine does not appear to engage in carbon-bridging behavior, presumably because of its very high electronegativity. **Struble *et al.*** (p. 57, see the Perspective by **Hennecke**) synthesized a rigid molecule, particularly well-poised to manifest fluoride bridging, and provide evidence for a fluoronium intermediate in a displacement reaction.

## A Supernova in 3D

When a star explodes, it expels matter at supersonic speeds. **Nikolić *et al.*** (p. 45, published online 14 February) used integral-field spectroscopy to obtain high-resolution, spatially resolved spectra of the shock waves associated with supernova 1006. The physical characteristics of the shocks around this supernova exhibit a strong spatial variation that suggests the presence of suprathermal (~10 to 100 keV) protons, which are the potential seeds for generating cosmic rays.



## MicroRNA Mechanism

MicroRNAs are small noncoding RNAs that regulate gene expression by binding complementary target messenger RNAs (mRNAs) and repressing their expression through repression of protein translation and mRNA degradation. **Meijer *et al.*** (p. 82) show that in a HeLa cell system mRNA degradation is a consequence of translational inhibition via the initiation factor eIF4A2.



Additional summaries

## From Grid to Place

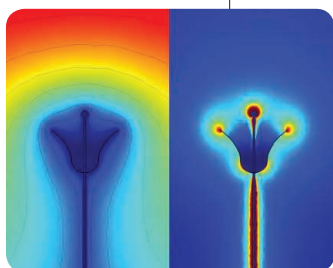
Grid cells are considered one of the key sources for place-cell signals in the hippocampus. The entorhinal circuit also contains other functional cell types, but it is unclear which project to the place cells of the hippocampus. **Zhang *et al.*** (p. 44, see the Perspective by **Poucet and Sargolini**) addressed this question using optogenetics and in vivo multi-electrode electrophysiology. Hippocampal cells received input from a broad spectrum of entorhinal neuronal cell types. Grid cells represented the biggest group of spatial inputs, but border cells, head-direction cells, and a large fraction of nonspatial cells also provided inputs. Thus, hippocampal circuits have local mechanisms for processing specific types of functional input from the entorhinal cortex to generate place-specific signals.

## Flowers and Bees Have “Sparks”

Plants and their pollinators have very intimate interactions, as emphasized by the many classic cases of coevolution among species of each.

Such close relationships require signaling between plant and pollinator. Coordination between plant signals and pollinator perception has been shown to exist in flower color, shape, and odor.

**Clarke *et al.*** (p. 66, published online 21 February) report the potential for a distinct mode of plant–pollinator communication, electric fields. Natural floral electric fields, which are impacted by visits from naturally charged bees, were easily discriminated by bees, based on their level, pattern, and structure, and improved the rate at which bees remembered the location of a nectar reward.



## Amorphous and More Active

The electrochemical generation of hydrogen from water could help in the storage of energy generated by renewable resources at off-peak times. However, catalysts for the slow step of this reaction, the oxygen evolution reaction (OER), are based on oxides of noble metals (iridium and ruthenium) that have limited abundance. A strategy for improving the performance of earth-abundant elements is to explore mixed-

metal oxides and to prepare these as amorphous phases. **Smith *et al.*** (p. 60, published online 28 March) developed a general method for preparing amorphous oxides, based on photodecomposition of organometallic precursors. Amorphous mixed-metal oxides of iron, nickel, and cobalt were more active than comparable crystalline materials and provided OER performance comparable to noble metal oxides.

## Adding to the Antiviral Arsenal

The envelope of influenza virus contains two immunodominant glycoproteins: hemagglutinin and neuraminidase (NA). Existing antivirals like zanamivir (Relenza) and oseltamivir (Tamiflu) target NA; however, the development of drug resistance is a problem. **Kim *et al.*** (p. 71, published online 21 February) now report a different class of NA inhibitors. NA catalyzes the removal of sialic acids from the surface of host cells to initiate entry. Discovery of a NA–sialic acid intermediate led to the production of sialic acid analogs that bound covalently to NA and inhibited its enzymatic activity. These

compounds showed activity against a wide variety of influenza strains, inhibited viral replication in cell culture, and were able to protect mice against influenza infection. Protection of mice was equivalent to protection seen from zanamivir. Moreover, the compounds showed activity against drug-resistant strains in vitro. These compounds represent a potentially useful addition to the arsenal of antivirals used to treat influenza infection.

## Putting Selenium in Proteins

The 21st amino acid, selenocysteine (Sec), occurs in the active site of many redox enzymes. Its cognate transfer RNA (tRNA) is first loaded with Ser by seryl-tRNA synthetase and the Ser-tRNA<sup>Sec</sup> is then converted to Sec-tRNA<sup>Sec</sup>. **Itoh *et al.*** (p. 75) determined the crystal structures of the selenocysteine synthase, SclA, that is responsible for this conversion in bacteria, alone and in complex with tRNA. The decameric SclA complex binds to 10 tRNA<sup>Sec</sup> molecules. The structures, together with biochemistry, show how SclA discriminates tRNA<sup>Sec</sup> from tRNA<sup>Ser</sup>, give insight into the mechanism of catalysis, and show that decamerization is essential for function.

## Silencing Transposons

Eukaryotic DNA is packaged onto nucleosomes, which are composed of four core histones (H2A, H2B, H3, and H4). Chromatin also contains a fifth histone, H1, which binds to both the core particles and the “linker” DNA that joins adjacent nucleosomes, where it helps chromatin to fold into higher-order structures and generally silences gene expression. In *Drosophila* germline and somatic cells and tissues, **Lu *et al.*** (p. 78) found that the repressive function of H1 in vivo was directed toward transposable elements and independent of small RNA silencing pathways. Instead, H1 acted through direct recruitment of the histone methyltransferase Su(var)3-9, which methylates histone H3 lysine 9, a repressive histone mark.

## Archaea Powered by Rocket Fuel

Perchlorate is a ubiquitous chlorine-based compound that forms naturally in the atmosphere. It is only present in large deposits in a few locations, such as the Atacama Desert in Chile, which has been assumed to be because perchlorate-reducing bacteria normally degrade it into chloride and oxygen. **Liebensteiner *et al.*** (p. 85; see the Perspective by **Nerenberg**), however, found that an archaeon, *Archaeoglobus fulgidus*, can also reduce perchlorate. The archaeal perchlorate reduction pathway shares limited similarity with bacterial perchlorate reduction: Instead of producing chloride and oxygen, enzymatically produced chlorite reacts with sulfide to produce oxidized sulfur compounds. Because hyperthermophilic anaerobic archaea similar to *A. fulgidus* are thought to have been among the first complex organisms to evolve on Earth, they may have started creating oxidized conditions in some habitats before the emergence of oxygen-generating photosynthesis.

## Neuronal Transposons

Transposons comprise a hefty chunk of the *Drosophila* genome and, unregulated, can generate mutations; thus, mechanisms exist to suppress transposon activity, particularly in the germline. **Perrat *et al.*** (p. 91) investigated transposon motility in neurons of the *Drosophila* brain. The mushroom body of the brain, responsible for olfactory memory, contains several different types of neurons. One class of neurons, the  $\alpha\beta$  neurons, exhibited increased transposon mobility, which generated increased neuronal diversity.

CREDIT: CLARKE ET AL.





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## Climate Change Conversations

THE THOUSANDS OF PRESENTATIONS AT NEXT WEEK'S MEETING OF THE AMERICAN CHEMICAL SOCIETY (ACS) in New Orleans exemplify one of the many ways scientists converse among themselves about the most recent advances in science. Science and technology continue to reshape the world we live in, and appreciating how these changes, both intended and unintended, come about is a necessity for all citizens in a democratic society. Scientists have a responsibility to help their fellow citizens understand what science and technology can and cannot do for them.

Communicating the science of climate change provides one example where the scientific community must do more. Climate change affects everyone, so everyone should understand why the climate is changing and what it means for them, their children, and generations to follow. Scientists are already members of groups that can facilitate this communication: neighborhoods, school boards, religious groups, service clubs, political organizations, and so on. These groups present opportunities to engage in respectful conversations on climate change and on the policies and actions that individuals, communities, and nations might take to mitigate and adapt to what is happening to our planet.

We know that the concentrations of greenhouse gases in Earth's atmosphere are higher and increasing faster than at any time in the past 1 million years.\* The average temperature of Earth is increasing, ice is melting, oceans are acidifying, and extreme weather events are more frequent. Human activities, principally the combustion of fossil fuels, are a major source of greenhouse gases and a major driver of climate change. To share this knowledge with the public and be credible as a "scientist-citizen," a scientist must acquire a good grasp of the science of climate change.

In recent years, U.S. scientific institutions and societies, including the National Academies, Environmental Protection Agency, National Aeronautics and Space Administration, and American Institute of Physics have prepared Web-based materials on the science of climate change suitable for communicating with the public.† Last year, the ACS released a Climate Science Toolkit on greenhouse gases, atmospheric and planetary warming, and Earth's energy balance, among other topics.‡ The Toolkit provides a succinct intellectual foundation at an introductory level that can be a guide to more extensive resources. Some of the materials are in forms (such as slide shows) that scientists may use to present this subject to the public, and there is a series of brief narratives designed to help scientists initiate informal conversations with others. Implicit in this resource is the message that the world must make adaptations to changes that have already occurred and that reducing emissions is required to avoid a warmer planet. Scientist-citizens can stress how lifestyle decisions that reduce energy consumption are actually meaningful steps. Supporting elected officials who promote policies and practices aimed to decrease the effects of global warming is another step that individuals and citizens' groups should take.

F. Sherwood Rowland was a central figure in the late-20th-century controversy about the effect of chlorofluorocarbons on stratospheric ozone. For years, he engaged audiences ranging from students to members of the U.S. Congress. As an exemplary scientist-citizen, his focus eventually led to the worldwide ban on these compounds. Rowland spoke to all scientist-citizens when he asked: "Isn't it the responsibility of scientists, if you believe that you have found something that can affect the environment, isn't it your responsibility to do something about it, enough so that action actually takes place?... If not us, who? If not now, when?"§

We pose these same questions and ask you to join the conversations now.

— Bassam Z. Shakhashiri and Jerry A. Bell



10.1126/science.1238241

\*[www.copenhagendiagnosis.com](http://www.copenhagendiagnosis.com). †<http://nas-sites.org/americasclimatechoices/sample-page/>; [www.epa.gov/climatechange/](http://www.epa.gov/climatechange/); [climate.nasa.gov/evidence/](http://climate.nasa.gov/evidence/); [www.aip.org/history/climate/index.htm](http://www.aip.org/history/climate/index.htm). ‡[www.acs.org/climate-science](http://www.acs.org/climate-science). §<http://www8.nationalacademies.org/onpinews/newsitem.aspx?RecordID=03122012>.

## EVOLUTION

### Cicada Cycles

The bizarre life cycle of the cicadas (genus *Magicada*) of eastern North America entails many years spent underground as larvae, before emerging synchronously as adults for a few brief weeks of sexual activity. Southern species have 13-year life cycles, whereas northern species have 17-year cycles. At any location, there are generally three species groups, all of which are synchronized with each other. The species groups are thought to have diverged allopatrically (i.e. geographically separated) before becoming sympatric as they are now. Several selective factors leading to the evolution of these life history patterns have been hypothesized, but in the absence of comprehensive phylogenetic data, they have proved difficult to test. Sota *et al.* have now addressed this gap, by conducting a thorough phylogenetic analysis of all known *Magicicada* species using nuclear and mitochondrial DNA markers. The resulting combination of phylogenetic and geographical patterns suggests a complex history of evolution—some of it quite recent—accompanying the habitat shifts in North America during the Pleistocene glacial cycles. The results are consistent with the allopatric divergence model and show that the splits into 13-year and 17-year species occurred multiple times within each species group. It appears that selection favored the evolution of synchronized population cycles between invading and resident species, simultaneously affording invaders protection from predation and avoidance of the adverse reproductive consequences of low population density. — AMS

*Proc. Natl. Acad. Sci. U.S.A.*, **110**, 10.1073/pnas.1220060110 (2013).

## IMMUNOLOGY

### Feeding Hungry T Cells

In order to mount productive responses against pathogens, T cells need to be fed. That is, the metabolic demands of T cell increase upon activation and as a result, T cells need to take up more amino acids. How this process is regulated, however, is not well understood. Sinclair *et al.* investigate and find that the System L amino-acid transporter, Slc7a5, which mediates the uptake of large neutral amino acids (LNAAs) such as leucine, was required for T cell activation in mice. In response to activation, T cells increased their expression of Slc7a5 and their uptake of LNAAs in a Slc7a5-dependent manner. T cells from mice with a T cell-specific deletion of Slc7a5 did not proliferate well or acquire effector cell functions in response to activating stimuli. This was correlated with a failure to activate the serine-threonine kinase complex mTORC1 and to induce protein

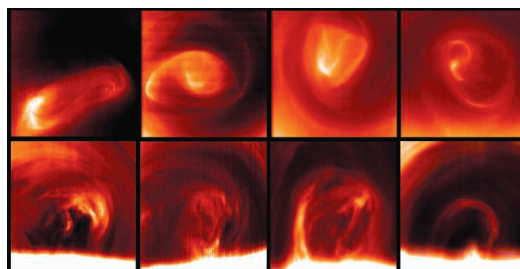
expression of the transcription factor c-Myc, both important components required to mediate the energetic demands of activated T cells. — KLM

*Nat. Immunol.* **14**, 10.1038/ni.2556 (2013).

## PLANETARY SCIENCE

### Vortex Complexity

The European Space Agency Venus Express spacecraft has been orbiting the second planet from the Sun since April 2006. Understanding



its atmosphere has been one of the main goals of the mission. Using data from the Visible and Infrared Thermal Imaging Spectrometer onboard the spacecraft, Garate-Lopez *et al.* examined the planet's south polar vortex by measuring cloud motions at two different altitudes. The data show that the vortex is long-lived, but its dynamical behavior and thermal structure are more complex than previously thought. — MJC

*Nat. Geosci.* **10**, 1038/geo1764 (2013).

## CELL BIOLOGY

### Polo's Game in Meiosis

The protein kinase Polo functions to promote cell division and is overactive in some cancer cells. Bonner *et al.* demonstrate a distinct role of the enzyme in regulation of meiosis: In this case, cells that fail to inhibit activity of Polo show improper chromosome segregation in meiosis or complete failure of meiosis. The protein Matrimony (Mtrm) directly interacts with Polo and appears to quench its activity. It appears that this may be the primary role of Mtrm in the cell, because the genetic effects of loss of Mtrm can be restored in cells in which Polo is also depleted. Phosphorylation-dependent binding of Polo to its targets is well characterized, but the interaction with Mtrm occurs in a different manner that may be more characteristic of Polo's interaction with its regulatory partners. Two domains of Mtrm appear to trap Polo and restrain its activity. Both proteins accumulate during meiosis I, and if Mtrm is lacking, proper meiosis is not achieved. — LBR

*Proc. Natl. Acad. Sci. U.S.A.* **110**, E1222 (2013).

## PHYSICS

### Purely Magnetic Impurities

Magnetically doped topological insulators (TIs) have been predicted to exhibit a host of exotic properties stemming from the breaking of time-reversal symmetry caused by magnetic impurities. However, dopants such as iron or manganese also have a charge effect, which distorts the original electronic structure and makes it difficult to isolate the effects of magnetism. Schlenk *et al.* devised a procedure for magnetic doping that aims to circumvent this problem and which should help to provide insight into the effects of time-reversal symmetry-breaking in TIs. They deposited Fe atoms on the surface of cold samples of the topological insulator Bi<sub>2</sub>Se<sub>3</sub> and then annealed them at much higher temperatures. Scanning tunneling microscopy indicated that the annealing caused the



dopants, initially adatoms, to descend into the material; angle-resolved photoemission found the electronic structure, initially distorted by depositing the adatoms, to be restored after the annealing step. First-principles calculations further indicated that the Fe atoms were probably substituting Bi atoms, and that whereas adatoms acted as electron donors, the buried Fe atoms were either neutral or acceptors; the magnetic interaction with the host material was also found to be enhanced with respect to the case of adatoms. — JS

*Phys. Rev. Lett.* **110**, 126804 (2013).

## ECOLOGY

### Marsh Basin Dynamics

Coastal salt marshes—found at temperate and high latitudes—form on tidal flats. Such marshes are both ecologically important and highly biologically productive. Although often protected, their fragile nature and location make them particularly vulnerable to human



activities and climate change. Mariotti and Fagherazzi analyze marsh basins—rounded tidal flats surrounded by salt marshes—at three sites on the U.S. Atlantic coast, as enlargement of marsh basins can drive marsh loss. They develop a simple dynamic model for the morphological evolution of marsh basins and show that the model has a single unstable equilibrium point for basin size. Below this size, basins shrink, and above it, they continuously enlarge. Aerial photos taken 50 years apart at the three locations reveal that for particular basin locations, the marsh boundary will grow and the basin will shrink, whereas at other locations, all basins are shrinking. The model suggests that sediment supply is the primary controlling factor for marsh boundary growth or recession, and historical data from the three

locations, where river dredging has in some cases significantly influenced sediment supply, support this idea. — GR

*Proc. Natl. Acad. Sci. U.S.A.* **110**, 10.1073/pnas.1219600110 (2013).

## GEOCHEMISTRY

### Unlocking Sulfur's Secrets

The sulfur isotope signatures of sediments and aerosol particles provide a rich record of biological and chemical processes in modern environments and in the geologic record. For example, the ratio of two or more of sulfur's several stable isotopes can indicate whether it was enzymatically reduced by microorganisms or if it underwent photolysis in the atmosphere after exposure to ultraviolet (UV) radiation. There are myriad proposed mechanisms to explain the underlying reactions that induce isotopic fractionation; disentangling them from one another is critical for interpreting their preservation. In a series of photochemical experiments, Ono *et al.* demonstrate that the partial pressure of  $\text{SO}_2$  strongly influences the sulfur mass-independent fractionation, at least in part due to self-shielding from UV photolysis by other  $\text{SO}_2$  molecules with a different isotopic composition. The experimental isotopic patterns are consistent with sulfate aerosols generated in the stratosphere, where UV photolysis is mostly confined; however, around 3 billion years ago,

Earth's atmosphere may have been much more transparent to UV radiation, creating a more extensive record of mass-independent fractionation. Farquhar *et al.* present an explanation for why the sulfur isotope signal generated by sulfate-reducing microorganisms—which were thought to have been present at that time—does not overprint mass-independent fractionation signals generated in Earth's early atmosphere. Spot analysis of sedimentary pyrites from Archean rocks suggests that there were two distinct sulfur pools contributing to most isotopic signatures: one of soluble oceanic sulfate and one of zero-valent sulfur derived from atmospheric deposition. — NW

*J. Geophys. Res.* **118**, 10.1002/jgrd.50183;  
*Proc. Nat. Acad. Sci. U.S.A.* **110**,  
10.1073/pnas.1218851110 (2013).

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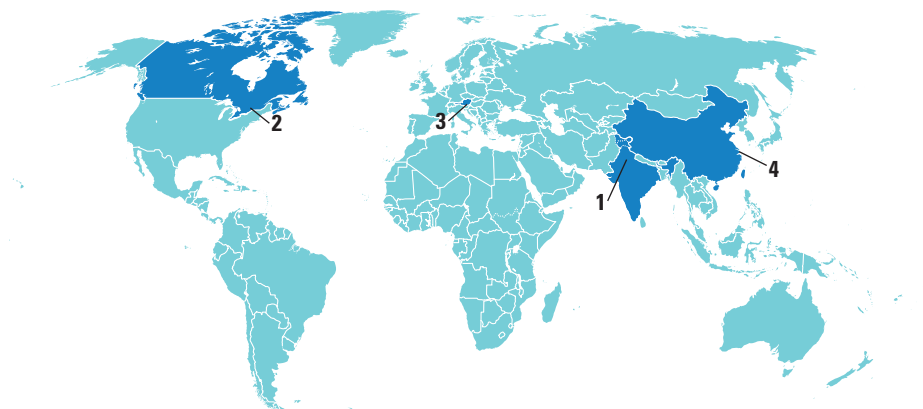
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## AROUND THE WORLD



New Delhi 1

**India Denies Cancer Drug Patent**

India's Supreme Court this week denied a patent sought 7 years ago by Novartis, the Swiss pharma company, for a crystal (pill) form of its celebrated anticancer drug Glivec (also known as Gleevec).

Groups pushing for low-cost medicines, such as Doctors Without Borders, cheered, while biotech and pharmaceutical groups warned of a possible chill in new drug development. The immediate impact will be to clear the way for cheap Indian versions of Glivec. According to *The Wall Street Journal*, the cost of treatment with Novartis's Glivec is about \$1900 per month compared with \$175 per month for generic drug versions made by Indian companies.

The key molecule (imatinib) was patented outside India in 1993, but India's patent office—which has not given Novartis a patent on any form of imatinib—rejected the company's request for a patent on the crystalline form in 2006 on the grounds that it wasn't a significant innovation. The Indian Supreme Court upheld that decision.

Novartis, noting that it provides Glivec free of charge to 95% of patients in India who receive a prescription for it, issued a statement saying that the court's decision "discourages innovative drug discovery essential to advancing medical science."

Ottawa 2

**Canada Withdraws From U.N. Desertification Treaty**

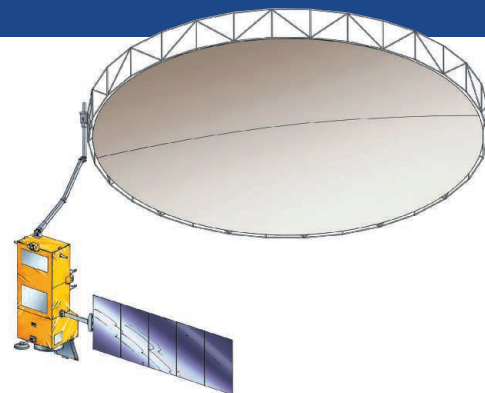
Canada's Conservative government announced last week that it is withdrawing from a U.N. convention to combat deserti-

fication signed by 194 other nations, fueling critics' concerns that the country is increasingly an outlier on climate change. Green Party leader Elizabeth May decried the move as a sign of the Conservative government's indifference to environmental protection. Prime Minister Stephen Harper said the move was about fiscal prudence, with only 18% of Canada's annual contribution of CAD\$350,000 "actually spent on programming" rather than bureaucratic measures, he told Parliament last week during a question period.



Noting that Canada was one of the first countries to sign onto the 1994 treaty, New Democratic and foreign affairs critic Paul Dewar said its decision to withdraw shows "that they either did not understand this convention, or they're willingly isolating Canada even more."

The government said Canada will not attend an international scientific conference on the convention taking place this month in Bonn, Germany. Dewar surmised that "maybe they were concerned because we hadn't been doing enough. Maybe they were concerned because they were going to be asked to do more. I don't know. ... It's a real head scratcher." <http://scim.ag/Candes>



Graz, Austria 3

**ESA Eyes Forest-Measuring Space Mission**

Biomass, a mission to measure the carbon content of the world's forests, is likely to get the go-ahead from the European Space Agency (ESA). Last month, the agency selected the €420 million mission over two other candidates, according to Volker Liebig, ESA's head of Earth observation. But the final decision rests with ESA's Earth Observation Programme Board, made up of representatives from ESA's 20 member states, which will meet in early May.

Biomass will use radio waves to assess the amount of material in a forest. Radio waves at different polarizations bounce off different parts of the forest canopy and soil; by measuring the power and phase of the reflected polarized beams, Biomass can calculate the mass, height, and structure of the forest.

Land-use change is one of the key sources of atmospheric carbon dioxide. Biomass will be able to measure the loss of carbon from deforestation and the effectiveness of reforestation. "It changes the game completely," says Shaun Quegan, chair of the Biomass mission advisory group. "We'll be able to see things we've never seen before."

Shanghai, China 4

**New Avian Flu Kills Two**

China's National Health and Family Planning Commission reported two deaths in Shanghai on 31 March from an avian influenza virus not previously identified in humans. The flu virus, H7N9, killed two men, ages 87 and 27, and infected a woman in nearby Anhui province.

It is unknown whether the outbreak originated in poultry or wild birds, says George F. Gao, deputy director-general of the China Center for Disease Control and Prevention (China CDC) in Beijing. China CDC researchers have sequences from two different strains of H7N9 and are now investigating the possibility of human-to-

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human transmission, Gao says. Although there is no evidence that the virus is airborne, he says, the possibility has not yet been ruled out.

The disparate locations of the three patients “would imply that the virus itself is also widespread in the animal population,” says Malik Peiris, a virologist at the University of Hong Kong. To avoid “a situation like H5N1, where the virus becomes entrenched,” he adds, any animal outbreaks must be quickly contained. <http://scim.ag/avianflu2>

## NEWSMAKERS



Gallo

### Three Q's

Hollywood director James Cameron donated his submersible *Deepsea Challenger*, which he rode to the bottom of the Mariana Trench last year, to

the Woods Hole Oceanographic Institution (WHOI) in Massachusetts last week. WHOI Director of Special Projects **David Gallo** talks about plans for the sub.

**Q: What's next for the *Deepsea Challenger*?**

**D.G.:** We're still beginning to understand exactly what's on that submarine. The cameras, the lights, the power—all of these things are advances for us.

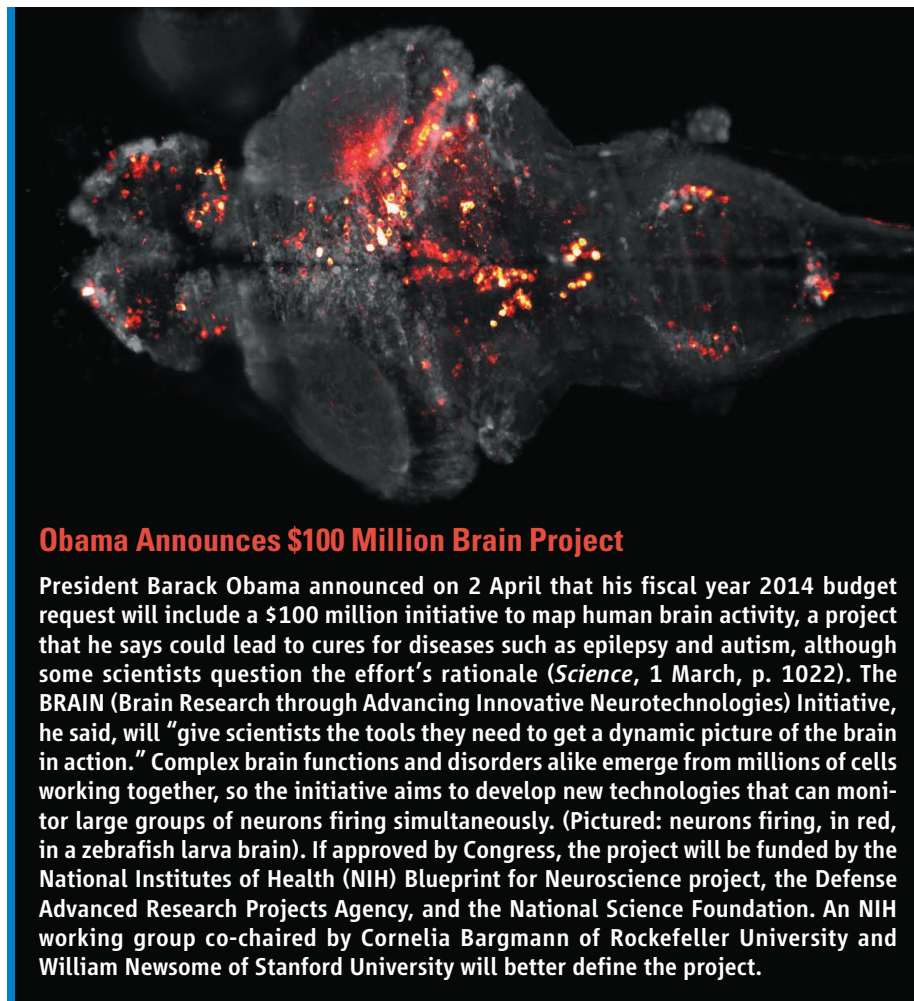
**Q: Which of *Deepsea Challenger*'s technologies will WHOI focus on in the near term?**

**D.G.:** Robotics. We've got a robotics system, the *Nereus* system, that's good to go. Before Jim made this offer, we were already heading to the deepest ocean. This is a real windfall for us, to be able to talk to Jim's team ... and find out what components of Jim's dream can be used on our robots. [And] we plan on using his cameras and lighting this June in the Caribbean to explore some of its very deepest spots.

**Q: Why use *Deepsea Challenger* rather than, for example, *Alvin*?**

**D.G.:** [*Deepsea*] *Challenger* opens up the entire ocean, even the deepest trenches [about 11,000 meters deep], to exploration. [*Alvin* can dive to 4500 meters.] There're all these different challenges about working deep, and Jim's done that homework for us.

We get hung up a lot on the tangible submarine, but this collaboration is as much about what we're going to do together in the future. ... We're looking at creating a



### Obama Announces \$100 Million Brain Project

President Barack Obama announced on 2 April that his fiscal year 2014 budget request will include a \$100 million initiative to map human brain activity, a project that he says could lead to cures for diseases such as epilepsy and autism, although some scientists question the effort's rationale (*Science*, 1 March, p. 1022). The BRAIN (Brain Research through Advancing Innovative Neurotechnologies) Initiative, he said, will “give scientists the tools they need to get a dynamic picture of the brain in action.” Complex brain functions and disorders alike emerge from millions of cells working together, so the initiative aims to develop new technologies that can monitor large groups of neurons firing simultaneously. (Pictured: neurons firing, in red, in a zebrafish larva brain). If approved by Congress, the project will be funded by the National Institutes of Health (NIH) Blueprint for Neuroscience project, the Defense Advanced Research Projects Agency, and the National Science Foundation. An NIH working group co-chaired by Cornelia Bargmann of Rockefeller University and William Newsome of Stanford University will better define the project.

whole new breed of scientific equipment to explore and open up the deep ocean. <http://scim.ag/WHOIChal>

### Marcia McNutt to Head *Science*



McNutt

Geophysicist **Marcia McNutt**, who stepped down as head of the U.S. Geological Survey (USGS) in February, has been tapped to be the new editor-in-chief of *Science*. McNutt will take over the editorship on 1 June from Bruce Alberts, who announced his retirement last year.

McNutt is no stranger to *Science*: She served on *Science*'s Senior Editorial Board, which helps set journal policy, from 2000 to 2009, an experience that she says will be helpful in her new job on several fronts. “It gave me a chance to understand at a high

### THEY SAID IT

**“It was becoming clear that there were people in NASA who would be much happier if the ‘sideshow’ would exit.”**

—Climate scientist and activist James Hansen in an e-mail to *The New York Times*, announcing his departure from NASA this week after 46 years at the administration.

level a lot of the decisions that the editor-in-chief is responsible for,” including the balance of content between news and research or between different disciplines.

Before she was appointed as director of USGS in 2009, McNutt was president and CEO of California's Monterey Bay Aquarium Research Institute for 12 years. <http://scim.ag/McNuttsci>



## Random Sample

## Antarctic Blue Whales Tracked by Their Songs

The *Remora* pulled to within 8 meters of the whale, trying to stay ahead to give marine mammal ecologist Virginia Andrews-Goff a good chance of attaching a satellite tag to the leviathan. “You’re ... looking/feeling for that correct spot, the correct angle and the correct moment during the whale’s surfacing to deploy the tag,” she says. “Afterwards I was in awe and feeling incredibly small.”

Andrews-Goff is part of a team of scientists from the Australian Antarctic Division in Kingston that has successfully managed, for the first time, to track the world’s largest creatures—the Antarctic blue whale—using only its sound. Researchers have long used the whales’ distinctive voices to study the animals, but developing a passive acoustic tracking system that could find them in real time was a trickier problem. Led by Brian Miller, the team last year tested hydrophone-bearing sonobuoys, very high frequency radio receivers, and custom software that successfully pinpointed pygmy blue whales by their songs at distances of more than 60 kilometers.

But the big success came this year, during the 47-day inaugural Southern Ocean trip of the Antarctic Blue Whales Project. Researchers were able to detect the “very deep and resonating song” of the blue whale from 600 nautical miles away, Andrews-Goff says. They recorded 626 hours of sound, analyzing 26,545 calls. The team also used those calls to triangulate the positions of the whales, so that they could col-



lect photo IDs as well as biopsy samples. They were also able to place satellite tags on two whales. The overall goal of the program, part of the international Southern Ocean Research Partnership, is to estimate the blue whales’ abundance and distribution, Andrews-Goff says. The information will be shared with the International Whaling Commission to aid conservation efforts.

## FINDINGS

## Hormone Therapy Breast Cancers Equally Virulent

Breast cancers linked to hormone replacement therapy (HRT) are no less deadly than those that strike women who are not taking hormones, a new study suggests. Published last week in the *Journal of the National Cancer Institute*, the study tackles a long-standing controversy: whether breast cancers associated with HRT are less likely to kill.

In 2002, the Women’s Health Initiative reported that results from a clinical trial showed that women on hormones who were diagnosed with breast cancer were at least as likely to die as those who got breast cancer while taking placebos. But observational studies suggested the opposite—that HRT-associated breast cancers have a better prognosis.

Rowan Chlebowski, a breast oncologist at Harbor-University of California, Los Angeles, Medical Center, and his colleagues tried to bridge that gap by studying more than 40,000 women who were part of a different effort, the Women’s Health Initiative Observational Study. The team found that survival after a breast cancer diagnosis was virtually the same in those who took

hormones and those who didn’t. But this, too, is unlikely to be the last word. One lingering question is whether there are differences in the biology of tumors that show up in women taking hormones.



## Sorting Through the Bits and Pieces of Plate Tectonics

Geologists long ago realized that slices of rock plastered onto North America—from southern Alaska to Nevada to western Mexico—are actually well-traveled terranes that began their geologic lives hundreds of millions of years ago as distant volcanic archipelagoes. But, using the latest seismic probings of the deep interior, researchers are

finding that the plate tectonic workings that delivered North America’s exotic terranes didn’t operate the way they thought.

Great slabs of ocean tectonic plate were once thought to deliver the crustal scraps to North America before sinking into the depths. But the positions and orientations of those slabs, now thousands of kilometers below Earth’s surface, do not jibe with that paradigm, say seismologist Karin Sigloch of Ludwig Maximilians University in Munich, Germany, and geologist Mitchell Mihalynuk of the British Columbia Geological Survey in Victoria, Canada, in this week’s issue of *Nature*.

Instead of the eastward-moving Farallon plate—now disappearing beneath the Pacific Northwest—delivering terranes to the continent, a westward-roaming North America must have plowed into them where they stood, building out the continent and driving up mountains along 6000 kilometers of North America. <http://scim.ag/WestUS>

## Science LIVE

Join us on Thursday, 11 April, at 3 p.m. EDT for a live chat on **genetic privacy**. How safe is your genome? <http://scim.ag/science-live>

**Getting to know you.**

Many scientists are studying the new coronavirus, named for the crownlike spikes on its surface.

## EMERGING DISEASES

# New Coronavirus Reveals Some of Its Secrets

On 19 March, a private Learjet touched down at the Munich airport. Inside the flying ambulance was a 73-year-old man from Abu Dhabi suffering from severe pneumonia. His family hoped German physicians might save his life.

They couldn't. A week later, the patient died at the Schwabing Clinic in northern Munich; his body was returned to the United Arab Emirates the next day. By then lab tests had shown that he was infected with a new coronavirus—the 17th known case worldwide and the 11th fatal victim. German officials have not revealed the man's identity, although that didn't stop the German tabloid *Bild* from claiming that he was “a sheik” from a “ruling family.”

Whoever he was, the man's final journey provided scientists another opportunity to learn something about a respiratory disease outbreak that is still surrounded by questions. As *Science* went to press this week, University of Bonn virologist Christian Drosten said he had already sequenced the genome of the virus and would soon publish a comparison with other known sequences that may shed some light on the pattern of spread of nCoV (for novel coronavirus), as the World Health Organization (WHO) has dubbed the pathogen.

Six months after the virus was first reported, scientists are convinced that there

are more people infected than the 17 known cases, some perhaps with cases so mild that they aren't seeking care, but they don't know how many; they assume the virus originates in animals, but they don't know in which species. There is almost certainly human-to-human transmission, but it's unclear how efficiently it occurs. The virus is a distant relative of SARS, the disease that terrified the world in 2003, but it's unknown if nCoV, too, has the potential to explode into a global health crisis.

Part of the problem is that the affected countries have released little information about the outbreak so far and appear reluctant to collaborate with foreign researchers eager to find out more (*Science*, 15 March, p. 1264). Saudi Arabia, for instance, has reported nine cases to WHO, but for some, there is no information about patient age, sex, place of residence, or circumstances surrounding the infection. “A lot of key epidemiological information is missing,” says virologist Vincent Munster of the Rocky Mountain Laboratories in Hamilton, Montana, which is part of the National Institute of Allergy and Infectious Diseases.

Since the virus emerged, scientists have published genomes of four strains isolated from patients, developed diagnostic tests, and set out to develop animal models—with the first successful attempt published online

this week by Munster's team in *The New England Journal of Medicine* (*NEJM*). In a paper published in the 14 March issue of *Nature*, a team led by Bart Haagmans of Erasmus MC in Rotterdam, the Netherlands, also identified the virus's receptor, a well-known protein called DPP4 that sits on the surface of cells deep inside the human lung.

So far, the virus appears to circulate primarily in the Arabian Peninsula. Beside the nine patients from Saudi Arabia, there were two from Qatar, and the first two confirmed cases are now known to have occurred almost a year ago during an outbreak in Jordan. (They weren't identified and reported until last fall.) Three U.K. residents were also infected—two fatally—in January and February, but the first one of those had traveled to Saudi Arabia and Pakistan before falling ill and presumably picked up the virus on the way. That two of his family members became infected suggests that the virus spreads from human to human—although apparently not very efficiently, because 135 of the patients' contacts weren't infected.

Munster's short paper published online in *NEJM* this week not only provides researchers with a way to test candidate drugs and vaccines, but also offers formal proof that nCoV—often called hCoV-EMC, a name given to it by Erasmus MC researchers who first sequenced it—is the cause of the human disease. For the study, Munster and his colleagues inoculated six rhesus macaques with the virus. They developed pneumonia within 24 hours, with symptoms such as fever, cough, and reduced appetite, and necropsy revealed bright red lesions throughout their lower respiratory tracts. (The team's pathologist called the severity of the disease—which varied from one animal to the next—“mild to moderate.”) The researchers reisolated the virus from the sick animals, thus fulfilling Koch's postulates, the classic set of criteria used to prove that a pathogen causes a disease.

Haagmans's group at Erasmus MC has tried the same experiment in cynomolgus macaques, but in that species the virus replicated poorly and didn't cause disease symptoms at all. Haagmans presented those findings at a Rotterdam meeting on 14 March, but says he wants to better understand why the animals were unaffected before publishing the data. It could be something species-specific, he says, or perhaps the monkeys were too young; previous work by the same group showed that older monkeys were much more susceptible to the SARS virus, a pattern that jibed with the



human outbreak. (Munster's rhesus monkeys were between 6 and 12 years old.)

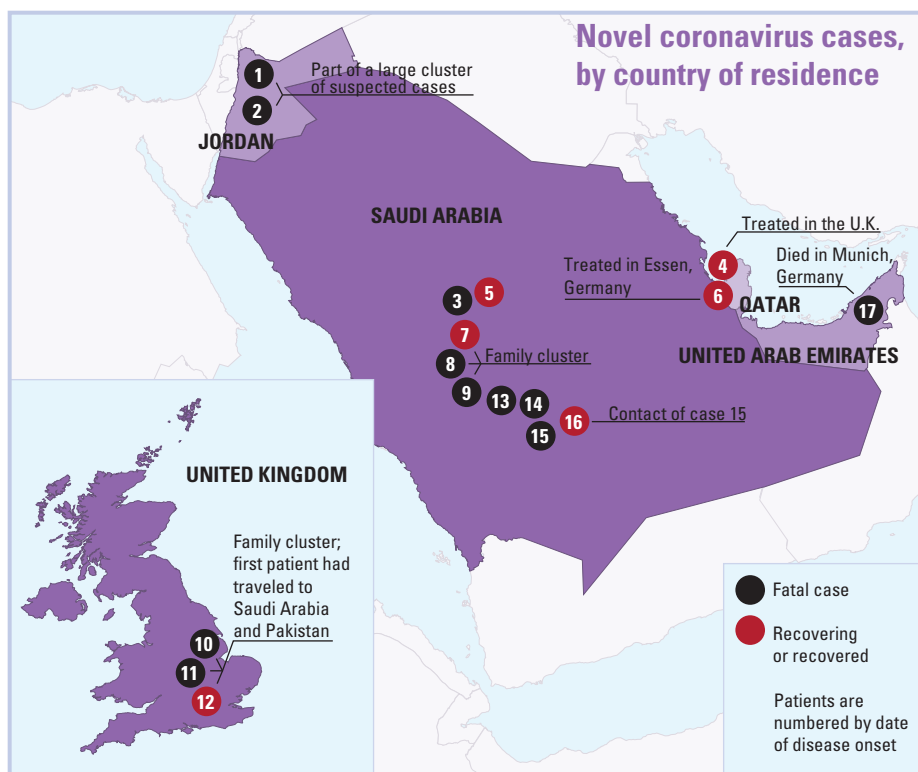
But few labs have monkeys, and they're expensive to work with; it's "really critical" that researchers also develop a small-animal model of infection and disease, says Matthew Frieman, a coronavirus researcher at the University of Maryland School of Medicine in Baltimore. Although the SARS virus does replicate in mice, and a mouse-adapted version makes them sick as well, Frieman and others have been unable to infect mice with the new virus. Munster has tried in vain with hamsters, and Haagmans's attempt in ferrets has failed as well. "We seem to have little luck with small animals," Haagmans says.

There are other ways of gauging the pathogenic potential of the new virus. A group led by Kwok-Yung Yuen at the University of Hong Kong tested whether it could infect a total of 27 cell lines from humans and animals, as a guide to which tissues and organs the virus might infect in the real world. In a paper published online by the *Journal of Infectious Diseases* on 26 March, they reported that nCoV replicated in 16 of the cell lines, more than any other known coronavirus—including human lung, kidney, intestinal, and liver cells. That might help explain why known cases of the disease have been so severe, the authors said.

But other researchers balked at that. In an accompanying commentary, Kenneth McIntosh of Boston Children's Hospital cautioned that studies of human cell lines—including some derived from tumors—often have little power to predict what happens in patients. "I think they drew too many conclusions," Frieman adds. In an e-mail to *Science*, Yuen says his group is well aware of the study's limitations, but writes that absent an animal model, the cell line approach "is all we can do at this moment."

Given the many questions about the epidemiology in the Arabian Peninsula, some researchers are hoping that the genomes of viruses found at different times and locales may yield clues about what is happening, an approach known as genomic epidemiology. So far, viral genomes from four patients have been published: the first reported case, a man who died in Jeddah in June and whose virus was sent to Erasmus MC; a patient who traveled to the United Kingdom in September for treatment; the first case in the U.K. family cluster earlier this year; and one of the Jordan patients, whose virus was obtained and sequenced by a U.S. Navy laboratory in Cairo.

In a recent paper in *Emerging Infectious Diseases*, a U.K. team published its genomic analysis of the first two cases; team member Andrew Rambaut, an evolutionary biologist



of the University of Edinburgh, then updated the analysis on his own Web site after two more genomes became available. The team found considerable variation between the four genomes, and calculations suggest that the virus has circulated since 2011, Rambaut says. In theory, it may have done so only in humans—assuming that many infections were mild and went undetected. But given today's travel patterns, it's unlikely that the virus would remain confined to the Arabian Peninsula if that were the case, Rambaut says. A more likely scenario is that it's circulating in animals and was repeatedly introduced into the human population, he adds—perhaps setting off short human-to-human chains of transmission now and then.

Drosten says that he soon plans to publish his own analysis, which will also include the newly sequenced virus from the Munich patient. In addition, Drosten says that he has an unpublished, partial sequence from a Qatari man who was treated at a hospital in Essen, Germany, last fall. (His group had huge trouble sequencing the genome because the samples contained very little viral RNA.) Drosten's analysis shows that the viruses from Qatar and the United Arab Emirates, which are geographically close, form a separate group—Drosten calls it the "Gulf clade"—from those in Saudi Arabia and Jordan. But he declined to speculate what, if anything, this says about the epidemiology.

Which animal species serves as the springboard to humans isn't clear, and there could be more than one. The viral sequences found so

far are closely related to bat coronaviruses, but bat-to-human virus jumps are rare because contact between the two species is uncommon. One notable exception is the Nipah virus, which is believed to cause human disease occasionally in Bangladesh when urine from fruit bats contaminates date palm juice. The alternative is that there is some intermediate animal host, Munster says.

Some anecdotal reports have suggested that livestock acted as that bridge species. German media have reported Clemens Wendtner, a physician at the Schwabing Clinic, as saying that the Abu Dhabi patient owned racing camels—a popular pastime among the Gulf's elite—and that he may have become infected when he visited them shortly before falling ill. The Qatari patient treated in Essen owned a camel and goat farm and told his German doctors that some of his goats had been sick before he fell ill himself; one of his animal caretakers also had a severe cough and was hospitalized.

Only epidemiology studies on the ground can bring more light to many of the questions, Munster says. Whether they are taking place in the four affected Middle Eastern countries is unclear even to WHO, which is trying to stimulate a concerted research effort. "I don't know if WHO has a full handle on all of the studies that are being done or are in the works," WHO spokesperson Gregory Hartl says—and as a human health agency, WHO is not involved in animal studies, he points out. "But we do need to know more about this virus." —MARTIN ENSERINK

## SPECIES EXTINCTION

## Fluttering From the Ashes?

For a science fair project when he was 13 years old, Ben Novak sketched out a recipe for resurrecting the extinct dodo. Many outgrow their wilder teenage passions. Not Novak. The molecular biologist at the University of California, Santa Cruz, is a key player in an effort now sailing into uncharted waters. “It’s my job,” Novak says, “to bring the passenger pigeon back to life.”

A noble mission? A misguided one? Or both? At a TEDx event last month at the National Geographic Society in Washington, D.C., experts explored the technical hurdles and ecological and ethical ramifications of reviving extinct creatures. Proponents say that they are driven, in part, to right historic wrongs. “If it’s clear that we exterminated a species, I think we have a moral imperative to do something about it,” says Michael Archer, a paleontologist at the University of New South Wales in Sydney, Australia, who is leading efforts to bring back two extinct Australian animals: the thylacine, a hyenalike marsupial, and a frog that brooded young in its stomach (*Science*, 22 March, p. 1371). The wow factor also plays in, especially when it comes to Ice Age megafauna such as the woolly mammoth. “The boy in me would love to see this majestic creature walking on the permafrost again,” says Hendrik Poinar, a molecular evolutionary geneticist at McMaster University in Hamilton, Canada.

But many are preaching caution. “We need to think very carefully before the saber-toothed cat is out of the bag,” warns Stanley Temple, a conservation biologist at the University of Wisconsin, Madison.

Billions of passenger pigeons darkened North American skies far into the 19th century. But the birds, sold for their meat, proved easy to slaughter; thousands could be captured in a single spring-loaded net. When the last of its kind expired in the Cincinnati Zoo in 1914, the passenger pigeon joined more than 200 bird species snuffed out in the past 2 centuries.

Last year, The Long Now Foundation, a nonprofit organization in San Francisco headed by Stewart Brand of the *Whole Earth*

*Catalog* fame, held a pair of meetings where scientists sketched out the feasibility of reviving the passenger pigeon. The technical hurdles are formidable. An option pursued for some extinct species—somatic cell cloning—has never worked in living birds, let alone dead ones. Today, says Michael McGrew, an embryologist at The Roslin Institute in Edinburgh, U.K., “We don’t have the technology to bring extinct birds back to life.”

Long Now is pursuing a radical strategy: Identify the genes underlying key passenger pigeon traits, such as its long tail or orange-



**Genetic life after death?** A century after the last passenger pigeon died, scientists are embarking on a controversial effort to resurrect the bird’s distinctive traits—if not the species itself.

colored breast, and splice them into a living relative like the band-tailed pigeon, which is common in the western United States. The first task is to reconstruct the passenger pigeon genome. Recently, Novak sequenced 500 million DNA base pairs—roughly half the genome—from three passenger pigeons stuffed in Troy, New York, in 1860. “We could get the whole genome from just these individuals,” says Novak, who is using the rock pigeon’s genome as a frame for aligning the sequence. The 1532 passenger pigeon specimens worldwide collectively represent

a wealth of genetic variation. Comparing the passenger pigeon and band-tailed pigeon genomes would pinpoint divergent stretches. The next step would be what geneticist George Church of Harvard Medical School in Boston calls “genome editing”: splicing passenger pigeon genes into the band-tailed pigeon genome using zinc finger nucleases. Trickier still is producing germ cells carrying the altered genome.

Even if a passenger pigeon began to take shape over successive rounds of genomic editing, there’s no telling whether the proxy would thrive. Long Now intends to use passenger pigeon puppets as parental simulacra. But puppets can’t teach young pigeons how to migrate. If the team gets that far, Novak says, it will paint the plumage of another migratory species, the homing pigeon, in the colors of their extinct cousins and release tagged passenger pigeons to the surrogate flock.

To Susan Haig, president of the American Ornithologists’ Union, the best avian candidates for resurrection are species that recently went extinct, did not migrate, and had a simple mating system, large clutch size, and minimal parental care. The passenger pigeon is not an ideal fit. Haig says she is “dying to see” the ivory-billed woodpecker, a 20th century extinction with enough extant habitat in the southeastern United States to stage a comeback.

The risks of revival are incalculable. “Maybe the passenger pigeon would be a wonderful vector for some nasty disease,” says Hank Greely, a bioethicist at Stanford Law School in California (see p. 32). Or its second incarnation could be a devastating invasive species. “No one wants to introduce an avian kudzu into the environment,”

Greely says. Another worry is that bringing back any extinct species—pigeon, frog, or mammoth—could undercut efforts to protect habitat for endangered species.

Like many scientists on the frontlines of preserving living species, David Ehrenfeld is lukewarm to the idea of bringing back dead ones. The “best expectation” of the passenger pigeon project and other exercises in “recreational conservation,” says the zoologist of Rutgers University in New Brunswick, New Jersey, may be to “recover lost traits worthy of study.” —RICHARD STONE



## NEUROSCIENCE

# How to Build a Dream-Reading Machine

"From the sky, I saw something like a bronze statue, a big bronze statue," the drowsy man told neuroscientists as he lay inside an MRI machine that moments earlier had been recording his brain activity as he dozed. "The bronze statue existed on a small hill. Below the hill, there were houses, streets, and trees in an ordinary way."

This surreal recounting of a just-interrupted dream—along with nearly 500 more descriptions gathered from the man and two other research subjects in a similar fashion—is the basis for an ambitious new study reported online this week in *Science* by a research team at the ATR Computational Neuroscience Laboratories in Kyoto, Japan (<http://scim.ag/THorikawa>). Utilizing these dream reports, the authors have, for the first time, successfully predicted images seen in sleep based exclusively on MRI scans of brain activity. Although researchers warn that we're still far from having a machine that can fully read our dreams, Robert Stickgold, a neuroscientist at Harvard Medical School in Boston who studies dreams, describes the work as "stunning in its detail and success."

Not long ago, the idea that you could "decode" what people were seeing, thinking, or dreaming about based on brain activity alone was "*Star Trek*, at best," Stickgold says. Over the past decade, however, ATR neuroscientist Yukiyasu Kamitani has been developing computer algorithms aimed at doing just that. In a 2005 paper in *Nature Neuroscience*, for example, he and colleagues described creating a computer program that can learn to associate the brain activity patterns recorded during functional magnetic resonance imaging (fMRI) with specific visual stimuli, in order to accurately predict at which of eight orientations of a grid a subject is looking.

To determine whether a similar approach could detect what sleeping people see, Kamitani and his colleagues recently recruited three volunteers to lie in MRI machines for 3-hour sessions over the course of 10 days. The loud banging caused by the vibration of an MRI machine's metal coils isn't conducive to slumber, but the volunteers wore headphones and found a way to doze. As each one drifted off, the researchers monitored the volunteer's brain activity with the

fMRI machine, which measures blood flow in the brain and is thought to reflect neuronal activity. They also used an EEG machine to track the brain's overall electrical activity.

Rather than waiting more than an hour for the people to enter rapid eye movement (REM) sleep, which is marked by long, often bizarre narrative dreams and temporary paralysis, the researchers took advantage of the frequent hallucinations that occur during the onset of sleep, called stage 1. People often don't know that they are asleep during

one category, for example, "ice pick," "key," and "plunger," were all grouped under the category "implement."

To train a computer program to associate broad categories of images seen in dreams with specific patterns of brain activity in the visual cortex, the researchers gathered photos on the Internet that corresponded to each category and recorded the three volunteers' fMRI activity as they viewed the images. Like other machine-learning technologies that can learn to identify hand-

writing based on subtle differences in penmanship, the program weeded out nonvisual brain activity that occurred during sleep and soon learned to associate specific brain activity "signatures" with different types of images, Kamitani says.

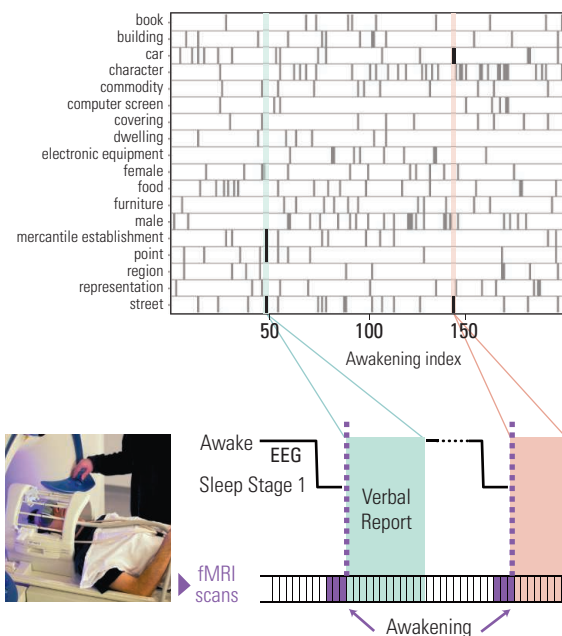
When the researchers applied the newly trained programs to a second round of dream-reporting by the same trio, coupled with fMRI monitoring, they were able to predict what the people had seen with 60% accuracy—a far higher rate than can be attributed to chance, Kamitani says.

"This is probably the first real demonstration of the brain basis of dream content," Stickgold says, describing the results as "incredibly robust." One exciting possibility, he says, is that the technique might reveal not only what we remember in our dreams, but also what we forget or fail to notice.

Technically, the images that the people in this study describe should not be called "dreams" at all, cautions Allan Hobson, a psychiatrist at Harvard Medical School who studies dreams. Instead, they are "hypnagogic hallucinations" with a different underlying physiology from the classic dreams that occur in REM sleep, he contends.

Researchers still find REM and stage 1 sleep mysterious, maintains ATR neuroscientist Masako Tamaki, a study co-author. "They may not be that different." She hopes that the technique will be applied in clinical sleep research and to help people who experience bad dreams. At a minimum, "it's nice to hear that what people report seeing when they're asleep is at least somewhat accurate," Stickgold says. "Up until this moment, there were no grounds on which to say we don't just make up our dreams when we wake up."

—EMILY UNDERWOOD



**Dream catcher.** Researchers collected reports of dreamed images from people awoken after sleeping in an MRI machine.

this stage, Stickgold says. (This is the same stage in which you might try to take the remote control away from a snoring companion and be grumpily refused, he adds.)

Once the three volunteers became accustomed to napping in the fMRI machines, they would fall easily into a light slumber, Kamitani says. Every 6 or 7 minutes, just as one began to drop into a deeper sleep, the researchers would see a ripple of EEG activity suggesting the likely presence of hallucinations. The researchers woke subjects, asked them to report anything they had seen, then told them to go back to sleep. After gathering roughly 200 of these reports from each subject, Kamitani's team extracted frequently repeated visual elements, such as "tree" or "man," from the reports and grouped them into approximately 20 broad categories tailored to each participant. In

# The Deep Earth Machine Is Coming Together

**Researchers studying how Earth's deep interior works are recognizing a new part connecting the depths to the surface, though the depths remain mysterious**

**EARTH IS AN ENGINE FUELED BY ITS OWN** heat. Now, after sharpening their view of the planet's rocky inner workings for almost a century, scientists are finally glimpsing how the Earth engine as a whole is working.

Since the plate tectonics revolution, researchers have recognized surface geology for what it is: a cold, rocky scum of continent-carrying, ocean-crust-covered tectonic plates. And the coldest, densest pieces of those ocean plates were clearly plunging into the barely yielding rocky interior, or mantle, toward the even hotter, molten core.

But the nature of what—ever might be carrying heat and material back toward the surface has been hotly debated for 40 years. Could towering plumes of hotter-than-normal rock be rising like lava lamp blobs from near the core? That could explain a range of geologic oddities, including the construction of monstrous piles of lava like Hawaii and mass extinctions seemingly linked to massive volcanic eruptions. Decades of study—imaging the mantle with seismic waves, divining the nature of the depths through geochemistry, and modeling the workings of the mantle the way meteorologists forecast the weather—now appear to be paying off.

"I'm finally off the fence," says seismologist Eugene Humphreys of the University of Oregon in Eugene. Humphreys thinks he can

"see," through seismic eyes, a plume that's been delivering the heat that drives eruptions around Wyoming's Yellowstone National Park. It is the first such feature—fed from the deepest reaches of the mantle—that looks like it will receive wide acceptance.

Along with recent work connecting plate tectonics to the deep interior, the recognition of such plumes is finally forging a strong link that spans the mantle from

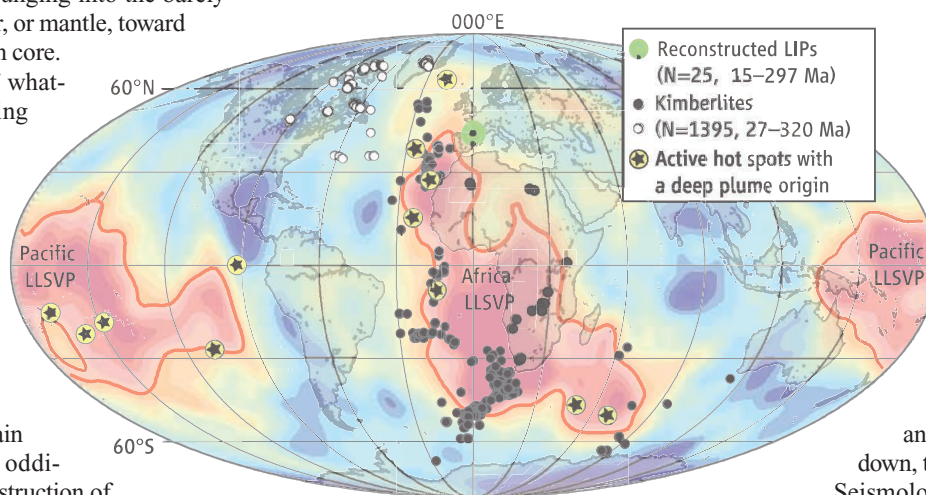
view, but some pieces of the Earth engine are yet to be labeled.

## Through a glass, darkly

Earth's interior didn't always seem so messy, or so interesting. By the middle of the last century, seismologists had divided the planet's 2900-kilometer-thick mantle into a half dozen layers on the basis of how seismic waves passed through the rock. Each layer,

as far as could be told, kept to itself. But then in the 1960s, plate tectonics came along. Central to the revelation of drifting continents was the realization that the planet's uppermost layer—the hundred kilometers or so of cold, rigid, crust-topped mantle constituting plates—was diving into deep-sea trenches and thus into the next layer down, the upper mantle.

Seismologists' next impenetrable boundary took longer to puncture. With more and better seismic records, researchers could trace descending plates, called slabs, much deeper using a technique called tomography. Because seismic waves speed up in cold rock, scientists can use them to form tomographic images of the descending slabs, much as they use x-rays in CT scans of the body. By the 1990s, seismologists could see some slabs struggling to pierce the supposed barrier between the upper mantle and the lower



**Birds of a feather.** Rising plumes likely connect huge volcanic eruptions (LIPs), diamond-pipe eruptions (kimberlites), and hot spots to two piles (pink, LLSVPs) on the bottom of the mantle.

bottom to top. Plumes of all sizes seem to rise from two huge piles of who-knows-what sitting 2900 kilometers down at the bottom of Earth's mantle embedded in a mystery layer hundreds of kilometers thick. The outline of an operating manual is coming into



mantle 660 kilometers down—and then, once through, heading for the bottom (*Science*, 31 January 1997, p. 613).

But what about plumes? Deep thinkers of the 1970s had invoked them as a way of explaining the Hawaiian volcanoes and a host of other volcanic hot spots from Iceland to Soma. Were seismologists of the 2000s catching plumes in their tomographic images of the mantle? “I would say no,” Humphreys says. A few seismologists would disagree, arguing that by dint of extra observations or special tomographic techniques they could image plumes stretching from the core-mantle boundary up to dozens of hot spots (*Science*, 22 September 2006, p. 1726).

Seismologist Jeroen Ritsema of the University of Michigan, Ann Arbor, sides with the doubters. That’s because he and colleagues have quantitatively considered just how hard it is to image plumes. “I hate to say this about my own work,” he says, “but tomography is not simple. You get very easily fooled.” Yong Keun Hwang of Michigan, Ritsema, and colleagues reported in *Geophysical Journal International* in August 2011 how a long list of factors works against seeing plumes: their expected narrowness; their relative warmth, which disperses seismic waves; the confounding effect of wave-bending variations in rock of the uppermost mantle; and on and on.

### Things looking up anyway

Plumes have long eluded seismologists, but after 40 years, solid-Earth scientists are coming to agree that they exist. “My take is not driven by observations but by fundamental theory,” says planetary physicist David Stevenson of the California Institute of Technology (Caltech) in Pasadena. “There’s heat coming from the bottom [of the mantle]; the natural way to take care of that is to have material peel away as plumes. I do believe this theoretical argument is quite a strong one.”

Mantle geochemists have their own line of argument supporting plumes. They have measured the trace elements, noble gases, and isotopes of rock brought to the surface in eruptions at hot spot volcanoes that have become ocean islands. They find that what goes down into the mantle—the ocean crust on slabs and sediments, mostly—can come back up eons later in the magma that builds ocean islands. And then there are the hot spot ocean islands that pop up one after the other in a line as an ocean plate passes over, like a smokestack blowing smoke into the passing breeze. “None of that is easy to do unless you accept plumes,” says geochemist Stanley Hart, a scientist emeritus from

Woods Hole Oceanographic Institution in Massachusetts. “I’m getting pretty close” to seeing plumes as unavoidable, he says.

In the past decade, even some geoscientists concerned mostly with the jostling of plate tectonics on the surface have embraced deep plumes. In a 2010 *Nature* paper, paleomagnetician Trond Torsvik and tectonophysicists Bernhard Steinberger of the University of Oslo and Kevin Burke of the University of Houston and colleagues summarized 10 years of work connecting some of Earth’s surface scum to those two piles of geocrud at the bottom of the mantle, one pile lying beneath Africa and the other under the South Pacific. Paleomagneticians measure the faint magnetic field locked into rocks when they form at the surface. They can then infer where a rock formed before plate motions carried it away to its present location.

In effect, Torsvik and his colleagues ran the plate tectonics part of the Earth machine backward (mathematically) for more than 200 million years. They wanted to see where, in relation to the interior, geologic features possibly related to plumes originally formed. Geochemistry and chain formation hint that 11 currently active hot spots may be deeply rooted in the mantle. Torsvik and his colleagues found that 10 of the 11 formed over or around the edge of a pile. Twenty-three of 25 so-called Large Igneous Provinces (LIPs)—huge piles of a few million cubic kilometers of lava like the Siberian Traps—erupted over the edges of piles. And 80% of kimberlite “pipes” through which diamond-bearing minerals were blasted to the surface from 100 kilometers down also erupted over a pile.

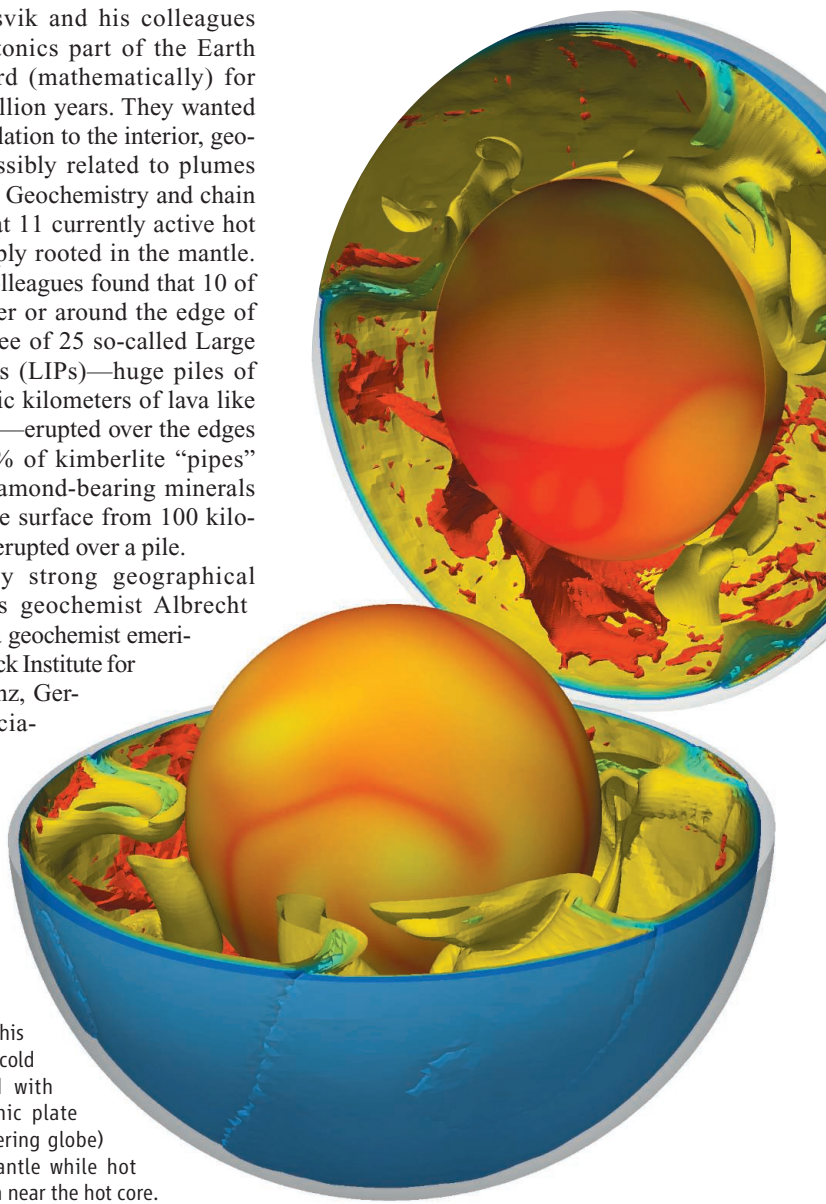
“That’s a very strong geographical association,” says geochemist Albrecht Hofmann, who is a geochemist emeritus at the Max Planck Institute for Chemistry in Mainz, Germany. “The association with the edges of two huge blobs strongly says there’s a causal connection.” The geodynamicists who run computer models simulat-

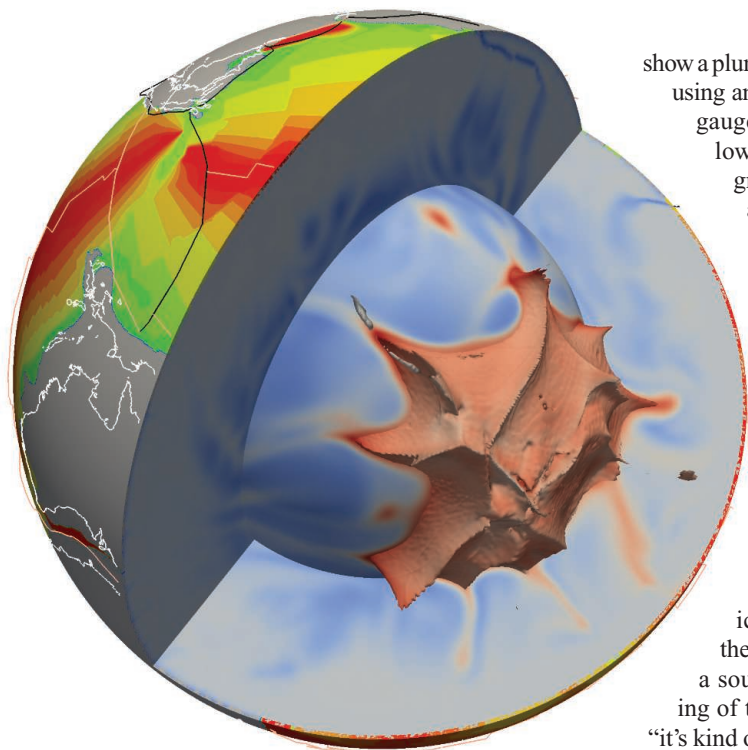
**All churned up.** In this computer simulation, cold slabs (yellow cored with light blue) of tectonic plate (dark blue when covering globe) descend into the mantle while hot plumes (red) rise from near the hot core.

ing Earth’s inner churning approve of that idea. “We can get plumes off the edges” of the piles, says geodynamicist Michael Gurnis of Caltech. “That seems reasonable.”

These three lines of evidence—geodynamical, geochemical, and geological—have moved the community toward the reality of plumes, seismological smoking gun or no. “There’s a comfort level with there being some material [at the surface] from the very deep Earth,” says Thorne Lay, a seismologist at the University of California (UC), Santa Cruz. And deep plumes are the only way anyone can imagine that happening.

There have been dissenters, of course. A small cadre of researchers has advocated, often energetically, nonplume explanations for supposed plume phenomena (see [www.mantleplumes.org](http://www.mantleplumes.org)). But “the





**Mantle shepherding.** In this computer simulation, cold descending slabs (blues) have herded dense mantle-bottom detritus into a pile or LLSVP (brown) from which hot plumes are rising.

plume deniers are fading,” says retired geochemist Hart. “The major forces are getting older, and none of the young guys are picking it up.” Plume critics aren’t writing peer-reviewed papers, he says, so “I don’t argue with them anymore; it’s a distraction.”

One relatively new and still emerging piece of evidence supporting plumes—published on 15 May 2012 in *Earth and Planetary Science Letters*—comes from seismologists, as a group the most cautious about accepting them. Brandon Schmandt of the University of New Mexico and his colleagues, including Humphreys, drew on observations from a unique source, the Transportable Array (*Science*, 25 September 2009, p. 1620)—an 800-kilometer-wide array of 400 seismometers stretched between the Canadian and Mexican borders—supplemented by other seismometers near Yellowstone. The combined observations provided the sharpest and deepest view by far of what lies beneath the Yellowstone hot spot.

In no uncertain terms, Schmandt and colleagues’ paper announced a hot mantle upwelling beneath Yellowstone. Their tomographic imaging used two different kinds of seismic waves to show extra-hot mantle rock extending down through the 660-kilometer-deep boundary between the upper and lower mantle to a depth of 900 kilometers, below which even their dense data set would not

show a plume even if it were there. And using another seismic technique to gauge the depth to the upper-lower mantle boundary, the group found the boundary—a temperature-sensitive change of mineral crystal structure or phase change—to be raised by 12 to 18 kilometers from its normal level across the same area that tomography shows to be extra hot.

Taken together, the results require a hot plume rising beneath Yellowstone, Humphreys says. And thermodynamics requires an origin near the core. “It’s all coming from a source associated with cooling of the core,” he says, adding, “it’s kind of sad that what it took was more measurements and not more cleverness, but that’s the case.”

Seismologist Barbara Romanowicz of UC Berkeley is impressed. She had never been convinced that any plume, including the iconic Hawaiian plume (*Science*, 4 December 2009, p. 1330), had been shown to rise from the deep mantle. In a 19 February interview with *Science*, she said she was, as Humphreys had been, still “sitting on the fence.” But after hearing Schmandt present the Yellowstone work at a seminar later that week, she came down off the fence. She found the two types of seismic data “quite convincing” that at least one deep plume rises into the upper mantle.

### About that crud on the bottom

If slabs are falling through the full depth of the mantle and plumes are rising from the bottom, then what, exactly, is going on down there at the deep end of the Earth engine? Seismologists have identified several different-looking sorts of geocrud sitting on the bottom. There are the two piles (technically called Large Low-Shear Velocity Provinces or LLSVPs, but who can pronounce that?). Seismology sizes up the piles as thousands of kilometers across and perhaps a few hundred kilometers high, made of somewhat different stuff from their surroundings.

Far smaller Ultra-Low Velocity Zones (ULVZs) tend to crowd around the piles but rise only 10 or 20 kilometers above the core-mantle boundary. And then there’s the core-enveloping D” (pronounced “D double prime”) layer topping out a few

hundred kilometers above the core.

Just what these seismic features might be hasn’t gotten much clearer since their discovery up to half a century ago. Mineral physicists squeezing and heating rock in the laboratory have lately found that iron-rich minerals—presumably enriched by iron from the core—might explain ULVZs, although some still favor partial melting of the rock. The mineral physicists also found a phase change that may explain the seismic upper boundary of D”, but what lies below it remains mysterious. And the composition of the piles is still anyone’s guess.

Stuck for the moment, researchers can turn to geodynamics. In the past few years, mantle modelers—including Paul Tackley of ETH Zurich in Switzerland; Shijie Zhong of the University of Colorado, Boulder; Allen McNamara of Arizona State University, Tempe; and Caltech’s Gurnis—have run simulations to see what would be the fate of anything dense enough to settle to the bottom of the mantle. In models of the mantle, slabs that are now descending in a ragged curtain from pole to pole provide the dominant driving force. Slabs churn the mantle from top to bottom, shearing any hapless slabs, sediments, or other tectonic detritus caught in midmantle into a 2900-kilometer-thick marble cake of rock.

In the models, the curtain of descending slabs tends to herd those slabs dense enough to reach the bottom intact and anything else already there into two antipodal structures near the equator. These structures bear a strong resemblance in shape and location to the current piles.

But what besides old slabs (it takes 100 million years or so to make the trip) might be down there? The leading candidate comes from the geochemists, who see it as a storage locker for rock left from the first 1% of the planet’s history. They find the barest of hints of such primitive stuff in the isotopes of noble gases from ocean islands and in the isotopes of the lanthanide element neodymium from ancient continental rocks (*Science*, 6 October 2006, p. 36).

Prospects for consensus on the bottom-most mantle are guarded. Seismologists will be markedly sharpening their tomographic imaging in the next 5 years, Romanowicz says, but sniffing out composition will remain a challenge. Mineral physicists are already pushing the envelope in the lab, although calculations of mineral properties from first principles may one day surpass lab work. And geochemists would like to find some more elements carrying clues to the nature of the deep mantle.

—RICHARD A. KERR

CREDIT: D. BOWER & M. GURNIS, CALIFORNIA INSTITUTE OF TECHNOLOGY



## MOLECULAR BIOLOGY

# 'Dead' Enzymes Show Signs of Life

**Pseudoenzymes can't catalyze chemical reactions, but researchers are discovering that they perform a variety of other cellular jobs**

Biologists were taken aback about a decade ago when they began to realize that a large fraction of human enzymes appear to be duds. That realization was a shock because we can't live without the enzymes that promote biochemical reactions. So how could so many of these critical molecules have lost their key function? And why does the body keep on producing apparently inert proteins?

One of the first indications that such "pseudoenzymes" are abundant came in 2002, when a research team combed through the newly available human genome sequence to identify all genes that encode protein kinases. These influential enzymes serve as switches in molecular circuits inside cells, transferring phosphate groups onto a variety of targets to help control a wide range of activities, such as metabolism and cell movement.

Of the 518 human protein kinases encoded by our DNA, about 10% lacked at least one of three key amino acids necessary for catalyzing the phosphate transfer and thus seemed to be inert, the scientists reported (*Science*, 6 December 2002, p. 1912). Although defunct enzymes had come to light before, the large number of these pseudokinases, as scientists dubbed them, was a shock, says team leader Gerard Manning, now director of bioinformatics and computational biology at Genentech in South San Francisco, California. "We thought we must have got it wrong."

They had it right, further studies have revealed. Almost every enzyme family includes seemingly inactive members, and in some clans, such as the sulfotransferases that swap sulfate groups, more than half the proteins encoded by our genome show signs of being catalytically compromised. And "dead" enzymes show up in organisms as diverse as bacteria, plants, and the mites that cause the skin condition scabies.

The pseudoenzyme genes that Manning and others identified are not degraded DNA. Cells make these proteins, and the DNA sequences for them have changed little over millions of years of evolution. That constancy suggests that the proteins have functions. "Biological systems don't bother keeping these proteins unless they are doing something important," says biochemist Patrick Eyers of the University of

Sheffield in the United Kingdom.

Sure enough, many supposedly inactive enzymes are gainfully employed in organisms—not as catalysts, but in a variety of other roles, researchers are now finding. Some help "true" enzymes catalyze biochemical reactions by forcing them into the correct shape. Others provide platforms where proteins can mingle. Still others join with receptors to help cells communicate, serve as bodyguards that escort proteins to new locations, or perform other tasks. "They turn out to be biologically really important,"

I were starting out again, I'd work on these [pseudoenzymes]," says biochemist Dario Alessi, also of the University of Dundee.

## A pseudoenzyme is born

To study pseudoenzymes, researchers first have to recognize them, but that isn't always straightforward. A candidate's amino acid sequence can indicate, but not confirm, that it's catalytically compromised. So researchers often determine the protein's 3D structure, test whether it can perform the expected biochemical reaction for an enzyme of its class, and conduct other experiments. Even then, the evidence is not always decisive (see sidebar, p. 27).

Despite the uncertainties, the consensus is that many proteins that look like enzymes primarily perform functions that don't involve catalyzing reactions. The majority



says protein chemist Susan Taylor of the University of California, San Diego. "They have been conserved for a reason."

Biochemist Daan van Aalten of the University of Dundee in the United Kingdom terms the DNA sequences encoding pseudoenzymes "the forgotten genes" because most researchers have overlooked them. But molecular biologists are now looking to the proteins encoded by these genes for insights into enzyme evolution. Drug designers hope to exploit them to create safer, more specific medications. And pseudoenzymes are causing biochemists to rethink some of their ideas about how conventional enzymes work. "If

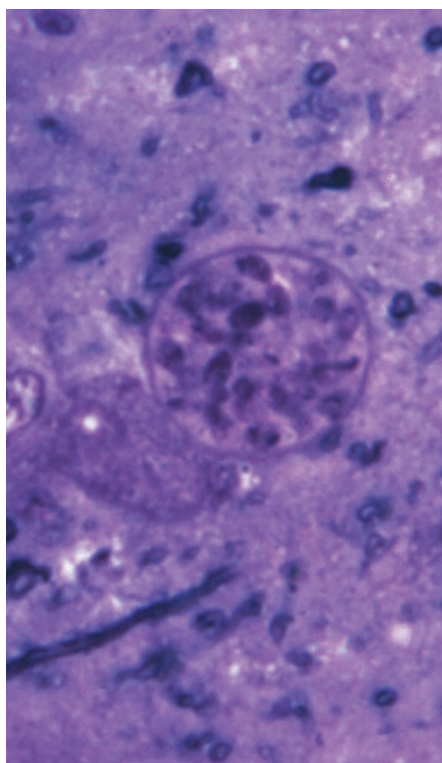
of these pseudoenzymes are closely related to active enzymes, and researchers have proposed two evolutionary explanations for such connections. Both involve a gene duplication at some time in the past.

In what researchers think is the more common scenario, the original and duplicated genes coded for an enzyme, and mutations corrupted the active site of one of the copies to produce a pseudoenzyme. "It's extremely easy to lose enzyme function because the catalytic residues [amino acids] are so well conserved," says computational biologist Janet Thornton, director of the European Bioinformatics Institute in Hinxton, U.K.

In some cases, the process may also operate in the opposite direction, Alessi says. “My theory is that an active kinase might have evolved from a pseudokinase.” In this scenario, the pseudoenzyme was already around, handling some noncatalytic cellular job, when its gene duplicated. Mutations then conferred catalytic ability on one of the versions, creating a working enzyme. A few dead enzymes do seem to have been the ancestors of living ones: Their functional descendants are recognizable because other members of the immediate family are not enzymes.

### Finding good help

Cells teem with perfectly good proteins, so why did they enlist defective enzymes to perform vital duties? Those pseudoenzymes that evolved from functional enzymes might have lost their catalytic ability, but they retained other talents. Before an enzyme instigates a chemical reaction, for instance, it grabs its target molecule, or substrate. Their inactive descendants often keep this binding ability, meaning they can home in on the protein for other purposes. Dead enzymes share other similarities with their active kin, notes cell biologist Matthew Freeman of the University of Oxford in the United Kingdom. They often hang out in the same tissues as their active counterparts and at the same time. Thus, a pseudoenzyme “is perfectly poised to have a regulatory role” and help control biochemical reactions, says biochemist Margaret Phillips of the Univer-



**Pseudoenzymes on the brain.** *Toxoplasma* parasites, shown here in a brain cyst, rely on pseudoenzymes to escape immune attacks.

STRAD $\alpha$  shows how a pseudoenzyme's binding ability enables it to regulate other enzymes. It latches onto and adjusts the activity of LKB1, a tumor suppressing kinase that's inactive in certain cancers. In a 2009 *Science* paper, Alessi, van Aalten, and

gests that “it's possible for a pseudokinase to activate a downstream kinase by binding it,” van Aalten says.

The iRhom pseudoenzymes that Freeman and colleagues study are also regulators, and they operate in two physiological contexts—antipathogen defense and sleep—that are so dissimilar “it's almost embarrassing,” he says. Their defensive role involves the secretion of TNF $\alpha$ , an inflammation-promoting molecule that various body cells emit when microbes menace. The release mechanism is involved. A cell first dangles a molecule of TNF $\alpha$  from its plasma membrane, and then an enzyme known as TACE snips the TNF $\alpha$  free. But to get into position to make the cut, TACE has to travel from the cellular organelle called the endoplasmic reticulum to the plasma membrane. Last year, Freeman's group and another led by immunologist Tak Mak of the University of Toronto in Canada revealed that iRhom2 binds to TACE in the endoplasmic reticulum and enables the enzyme to leave the organelle (*Science*, 13 January 2012, pp. 225 and 229). Without iRhom2, TACE doesn't depart, leading to reduced TNF $\alpha$  secretion and weakened immune defenses. Mice lacking iRhom2 are more susceptible to bacterial infections.

### Going over to the enemy

Pathogens also take advantage of pseudoenzymes. Phillips and colleagues have discovered, for example, that dead enzymes are crucial for *Trypanosoma brucei*, the parasite that causes African sleeping sickness in people. Pseudoenzymes increase the activity of two of the parasite's key metabolic enzymes by up to 3000 times.

One of the world's most widespread parasites, *Toxoplasma gondii*, also deploys pseudoenzymes, using them to help confound immune defenses of one of its hosts. *Toxoplasma* dwells in nearly one-third of the world's human population, usually without causing distress, but people aren't the only stopovers during the parasite's life cycle. “*Toxoplasma* is highly unusual in being able to infect virtually any bird or mammal,” says molecular biologist John Boothroyd of the Stanford University School of Medicine in California. Rodents, which are one of the parasite's hosts, fight back against *Toxoplasma* with proteins from the IRG family. They coat the capsules in which the parasites lurk, killing the organisms inside. To protect itself, the parasite releases a catalytically active enzyme, ROP18, that disrupts IRG proteins.

## Pseudoenzymes and their functions

| Name                   | Enzyme family          | Molecular action                                              | Putative function                                                 |
|------------------------|------------------------|---------------------------------------------------------------|-------------------------------------------------------------------|
| Deoxyhypusine synthase | Deoxyhypusine synthase | Adheres to metabolic enzyme                                   | Boosts activity of partner enzyme in <i>Trypanosoma</i> parasites |
| iRhom                  | Rhomboid protease      | Binds to and spurs destruction of growth factor-like proteins | Inhibits sleep in fruit flies                                     |
| Tribbles homolog 3     | Protein kinase         | Binds to and blocks signaling kinases                         | Impairs release of and response to insulin                        |
| STRAD $\alpha$         | Protein kinase         | Binds to active enzyme LKB1                                   | Controls partner enzyme's activity                                |
| TAB1                   | Phosphatase            | Joins protein complex                                         | Spurs phosphorylation of active kinase TAK1                       |

sity of Texas Southwestern Medical Center in Dallas. In many cases, Freeman says, researchers have established that pseudoenzymes regulate the reactions that involve their active relatives.

colleagues showed how STRAD $\alpha$  works. With help from a third protein, MO25 $\alpha$ , STRAD $\alpha$  locks LKB1 into its active pose, allowing it to perform its job (*Science*, 18 December 2009, p. 1707). The work sug-



## Dead or Alive?

The enzymatic competence of proteins can be difficult to judge, and some apparent pseudoenzymes have fooled researchers. Take CASK, a protein found in neurons that is one of the original “nonfunctional” kinases pinpointed by Gerard Manning, director of bioinformatics and computational biology at Genentech in South San Francisco, California, and colleagues (see main story, p. 25). Kinases typically swipe a phosphate group from the molecule adenosine triphosphate (ATP) and pass it to another protein. CASK lacks two key amino acids that researchers thought were necessary for transferring a phosphate, which suggested that it’s a prototypical pseudokinase.

But after determining the crystal structure of a portion of the protein (right), neuroscientist Konark Mukherjee, now at the Virginia Polytechnic Institute and State University Carilion Research Institute in Roanoke, and colleagues reported that CASK can use alternative amino acids to instigate the kinase reaction. In *Cell* in 2008, the team also revealed evidence from test tube experiments and cultured cells that suggests that CASK serves as

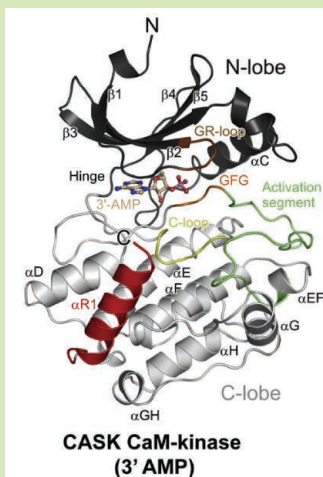
a catalyst, albeit a weak one. Whether that’s the biological role of CASK remains unclear, however, leaving its pseudostatus up in the air.

Researchers sometimes get similarly fooled into thinking a protein is an enzyme. Early biochemical studies suggested enzyme activity for integrin-linked kinase (ILK), a potential drug target because mutated versions promote cancer. Drug developers even began trying to identify inhibitors under the assumption that it was a functioning kinase, says structural biologist Jun Qin of the Cleveland Clinic Foundation in Ohio.

Yet when he and his colleagues figured out the 3D structure of a fragment of ILK attached to its activator, they found that ILK’s ATP-binding site was far from the part of the protein that would catalyze a reaction, making the phosphate handover unlikely. “We now know it has no kinase function,” says Qin, whose team reported its findings in *Molecular Cell* in 2009. Instead, buttressing its pseudoenzyme designation, ILK links to other proteins

to form a relay between a cell’s skeleton and receptors on its surface.

—M. L.



Last year, Boothroyd’s group and two others reported that a *Toxoplasma* pseudoenzyme called ROP5 partners with ROP18 in the fight against IRG proteins. ROP5 latches onto an IRG protein and clamps it in a shape that is vulnerable to attack by ROP18. Boothroyd says that he likens ROP5 to “the bully’s sidekick who holds the kid in the schoolyard while the bully beats him up.”

ROP5 helps ROP18 hit back in other ways, too. The group led by microbiologist L. David Sibley of Washington University School of Medicine in St. Louis found that ROP5 also holds ROP18 in its active shape so that it can neutralize the defensive proteins. It’s not surprising that *Toxoplasma* turned to a pseudoenzyme to assist ROP18, says Boothroyd’s co-author Michael Reese, a biochemist also at the Stanford University School of Medicine. The parasite is rife with dead enzymes—among the organisms whose genomes researchers have sequenced, *Toxoplasma* has the highest proportion of pseudokinases, Reese notes.

The parasites can carry more than 10 copies of the ROP5 gene that differ only slightly from one another, possibly revealing an evolutionary battle with their host’s immune system, Sibley says. You’d expect that mice would evolve slightly different IRG proteins that are better weapons against *Toxoplasma* and that the parasites would respond by tweaking the ROP proteins and

creating new versions. But mutations that modify ROP18’s amino acid sequence could undermine its function, so allowing ROP5 to change might be a safer strategy, Sibley says. Without a catalytic site than can be compromised, he notes, “it doesn’t matter if it’s peppered with a lot of mutations.”

### Getting practical

Given their ability to regulate active enzymes, pseudoenzymes have promise as drug tar-

**“If I were starting out again, I’d work on these [pseudoenzymes].”**

—Dario Alessi,  
University of Dundee

gets, some scientists suggest, although no such drugs are in use. Going after dead kinases, Evers says, may offer alternatives to traditional kinase inhibitors, a fast-growing drug class responsible for nearly \$11 billion in sales in the United States alone. Probably the most famous kinase inhibitor is Gleevec, which reached the clinic in 2001 and has proven successful against chronic myelogenous leukemia.

The drawback of such inhibitors is that because the kinase active site is so similar across enzymes, drugs that block one kinase

can interfere with others, triggering side effects. Gleevec, for example, can cause abdominal pain, nausea, fatigue, and other problems. But indirectly blocking an overactive kinase that has pathogenic effects by targeting its pseudoenzyme partner might leave other kinases unscathed. However, some remain skeptical that pseudoenzymes are worth aiming at, notes biochemist Mark Lemmon of the University of Pennsylvania. Researchers have a lot of experience designing drugs to block kinases, but devising one that disables a pseudokinase is a challenge because the traditional and well-studied enzyme active site isn’t the natural place to hit. “They could still be very important drug targets,” he says. “We just have to think differently” about how to inhibit them.

Even if pseudoenzymes haven’t yet made much of a practical impact, they’ve had an intellectual effect, according to Evers. “Pseudokinases have made us think, ‘We really don’t know so much about kinases,’” he says. As a result, biochemists are taking a second look at basic questions, such as how much activity an enzyme has to show to be considered functional. At a broader level, the discovery of this new category of inactive enzymes reveals how little researchers understand about parts of the genome, Freeman says. “I’m intrigued by the concept that there are a lot of genes that we know nothing about.”

—MITCH LESLIE



## LETTERS

edited by Jennifer Sills

## NextGenVOICES

## Results: Science Communication's Future

Ideally, how will scientists share their results with each other and the public in 50 years? In January, we asked young scientists to use their imaginations and send us their best ideas. We heard from nearly 150 readers, many of whom share a vision of a world in which scientific results are online, interactive, and easily accessible to both scientists and the public. A sample of the best responses can be found below. To allow for as many voices as possible, in some cases we have printed excerpts of longer submissions (indicated by ellipses) and lightly copyedited original text for clarity. To read the complete versions, as well as many more, go to [http://scim.ag/NextGen6\\_Results](http://scim.ag/NextGen6_Results).

## Submit Now: Science Time Travel

Add your voice to *Science*! Our new NextGen VOICES survey is now open:

**You can travel back in time to share one piece of scientific knowledge from today. Where do you go? Describe the date and place you choose, the information you share, and how it might change the course of history.**

To submit, go to <http://scim.ag/NextGen7>

Thanks to Jon Tennant at the Department of Earth and Engineering, Imperial College London, for submitting this question.

Deadline for submissions is 17 May. A selection of the best responses will be published in the 5 July issue of *Science*. Submissions should be 250 words or less. Anonymous submissions will not be considered. Please submit only once.

## NextGen Speaks

FEBRUARY 12, 2063. TODAY I UPLOADED my manuscript to the reviewing system of the General Platform. I chose my subsection such that it will end up with the right reviewers.... Luckily, manuscripts can't get rejected anymore, but I wonder



what remarks, suggestions, and initial ratings my paper will receive and how much I will need to revise. What's most exciting is whether after revision, my paper will be selected for a journal: From the General Platform,

journals make weekly selections of new and important papers that best suit the interest of their subscribers.... I hope my paper will be selected by a widely read journal. Maybe the journal will also publish an additional comment! Still, most important are the comments

and ratings readers will give my paper, since even without journal selection, it will remain available on the General Platform.... As the ratings are given anonymously, papers are really graded for their worth, and I will get a good sense of what my paper contributes to the field. Well, let's stop hoping and wait for the reviews!

**ANNELINDE R. E. VANDENBROUCKE**

Cognitive Neuroscience Group, Psychology Department, University of Amsterdam, 1018 XA, Amsterdam, North Holland, Netherlands. E-mail: [a.r.e.vandenbroucke@uva.nl](mailto:a.r.e.vandenbroucke@uva.nl)

IN THE NEXT 50 YEARS, I BELIEVE THAT BREAKTHROUGHS in visualization of huge data sets will revolutionize the way science is communicated, making results much more accessible to scientists and the public alike. Genomics gives a perfect example of where visualization may be heading. In coming decades, I believe that laypeople will be able to upload personal genomic data (which will soon be easily

affordable) onto a data-rich genome browser, which displays many levels of information in navigable format. The browser may, for example, present a three-dimensional view of a DNA helix, bundled onto histones and studied by proteins and polymerases, that may be uncoiled and queried by voice or touch. From any DNA region of interest, users will be able to toggle articles, blogs, and social media posts, as well as demographic, lifestyle, and disease-related histograms. Portions

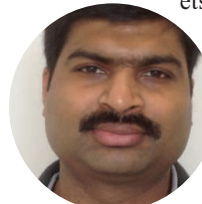


of the browser might light up to announce new scientific findings, with confidences of individual results noted via some standardized criteria. Software developers and scientists will provide various specialized viewer plugins that people can run as apps. All this will give the public unprecedented access and exposure to cutting-edge science. Big data browsers will by no means be limited to genomics; we already see their prototypes in tools such as Google maps, which may soon support toggling and viewing of geological and climate data. These tools will fundamentally alter the way scientific findings are disseminated and will go a long way toward improving trust and dialogue between scientists and nonscientists.

**MATTHEW OBERHARDT**

Departments of Computer Science and Molecular Biology/ Biotechnology, Tel Aviv University, Tel Aviv, 69978, Israel. E-mail: [mattoby@gmail.com](mailto:mattoby@gmail.com)

ONCE SCIENTIFIC FINDINGS ARE SUBMITTED to communication portals, machine intelligence will distill scientific results into packets relevant to every demographic: other scientists, informed citizens,



teachers and students, and children. These packets will be made into annotated, high-definition, holographic

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A new era for  
insomnia treatment?

36

Modeling solar  
magnetism

42

interactive, multimedia presentations appropriate for each demographic. Specialized devices, equipment, methods, and procedures will be virtually simulated to create a tactile experience for the audience, especially for education and training. Successive publications get consolidated into a timeline of science communication, both at the individual scientist level and at the level of the larger area/field. Conferences for specialized and general audiences will bring people together, but virtual participation will be the norm.

**ANIL KUMAR CHALLA**

Department of Genetics, University of Alabama at Birmingham, Birmingham, AL 35294-0024, USA. E-mail: anilkchalla@gmail.com

IDEALLY, AS SCIENTISTS, WE CAN HOPE FOR A greater scientific understanding by the general public as myths are replaced by facts. This should lead to a greater allocation of space in newspapers, television, Internet webzines, and social media for science. More knowledgeable discussion among the general public would lead to an improvement in scientific research and funding....

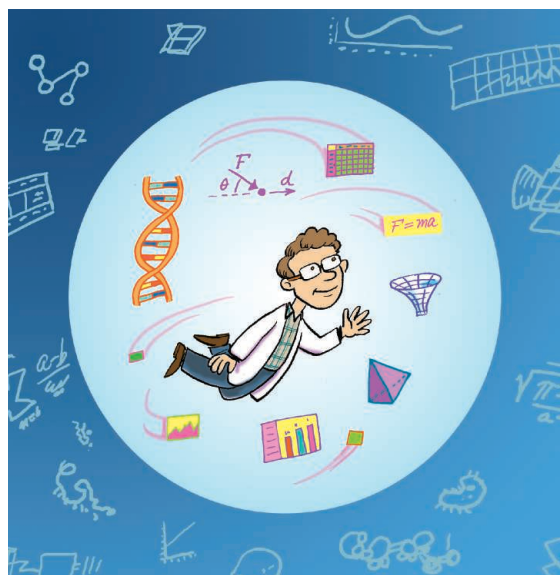
**K. CHRISTIAN KEMP**

Center for Superfunctional Materials, Department of Chemistry, Pohang University of Science and Technology, Pohang, 790-784, Korea. E-mail: ckemp@postech.ac.kr

...IN 50 YEARS' TIME...SCIENCE COMMUNICATION will be in some form of social media, most likely, personalized blog posts that are updated and edited by individuals, or highly targeted short message links that automatically seek out the right audience at the right time, like heat-seeking missiles, to achieve highest impact....

**FREDROS O. OKUMU**

Environmental Health and Ecological Sciences Thematic Group, Ifakara Health Institute, Dar es Salaam, Tanzania. E-mail: fredros@ihi.or.tz



...RESEARCHERS SHOULD NOT HAVE TO WAIT to share results (even negative ones) until the entire project is established with enough data to be worthy of publication in a journal. Experiments and their results could be posted one by one in a forum or database-like environment, with proper acknowledgment and enough protection for the respective researcher.... Groups working on similar projects could communicate, share thoughts, generate ideas, or even combine efforts, hence accelerating the project and the publication process.... The real essence of scientific research would be captured and tailored to the fact that in this day and age, the world has no boundaries.

**YASMINE MUSTAFA MOHAMED**

Department of Biology, American University in Cairo, New Cairo, 11835, Egypt. E-mail: y\_mustafa@aucegypt.edu

...PERHAPS SCIENCE COMMUNICATION should completely abandon the research paper as we know it. Ideally, scientific communication would be a single, completely open, digi-pedia combined with a simple rating system and powerful search engine.

Textbook-like, hierarchical organization of content would end with links to primary data of any type, topic forums, and tutorials, all unrestrictedly posted by the user. Articles, experimental techniques, primary data, researchers, and institutes would each be subjected to mass ratings by the reader.... Points of contention would be quantified and used as an additional sorting factor to identify critical questions. Negative data would be communicated and confirmed. Ratings would act as guides for rewarding achievement in all aspects of the scientific process....

**SCOTT ALLEN LACADIE**

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THE UBIQUITY OF SMARTPHONES, robotic assistants, and wearable sensors will usher in an era of Big Data never before seen: Scientific publications won't be written

so much as aggregated from an unceasing supply of images, assays, raw numbers, and composite text edited with e-mail-screening software. Once reports are "published" (physical printing will have mostly ceased, so this word sounds arcane), you'll be able to view an AutoCAD-like experimental layout using any heads-up display, perhaps projected onto your lab goggles. When you stare at an empty wall or table, a full experimental manifold will expand in front of you—outlines tracing equipment, animals, and reagents, showing experiments in real time. Tactile gloves will provide haptic feedback in real time; you'll "touch" and manipulate the objects you see. Dedicated media professionals will incorporate the best experimental animations into an annual movie, available for download at any public Sci-terminal. Digital awards, like today's Oscars or Emmys, will signify the high-quality research, driving tenure decisions and funding.

**MICHAEL A. TARSELLI**

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...IN 50 YEARS, WE CAN diversify our audience by refocusing our nations' education systems. We can integrate the current methods of sharing results into curricula of secondary schools and undergraduate programs.... Researchers must take on an additional responsibility: They must orient their published results toward not only the knowledgeable but also the curious. They need not alter the technicality of their results; instead, they can draft secondary articles focusing on the applications of their research.... Teachers and professors who keep their students up to date on current scientific developments will foster a sense of determination and purpose in the next generation of scientists. Within 50 years, several generations will have grown up surrounded by scientific innovation. The general population will finally constitute the majority of a scientist's audience.

**VIKAS ARAGAM**

College of Arts and Sciences, University of Pennsylvania, Philadelphia, PA 19104, USA. E-mail: vikasar@sas.upenn.edu

...I ENVISION A FUTURE

of...scientists who as easily construct an exquisite phrase as a well-planned experiment. Ideally, we will be a generation of savvy bloggers, social media personalities, and passionate public speakers. We will do this out of interest, but also by necessity. The fate of research funding rests on our ability to communicate science topics widely and well, both to our peers and to the public. We cannot afford to let other people speak for us; we must be trained to speak for ourselves.

**ERIN E. COFFEY**

Department of Anatomy and Cell Biology, Neuroscience Graduate Group, University of Pennsylvania, Philadelphia, PA 19104, USA. E-mail: coffey@mail.med.upenn.edu

...EVERY LABORATORY WILL HAVE FAN PAGES ON social media platforms, and scientists will share their insights

on a daily basis with the general public on current issues. I see, in 50 years, a situation where crowd funding of research is the main funding instrument that will reward scientists with stronger public engagement skills....

**PATRICK KOBINA ARTHUR**

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GOVERNMENTS SHOULD buy all the major publication houses and release their information for free, while maintaining the journal structure at public expense. The costs would be trivial relative to the tax revenues captured from the growth spurred by the open-access policy. I cannot think of a better way for the world's governments to spend a few billion dollars up-front and several hundred million per year.

**CHAD BRICK**

Sumitomo Bakelite Corporation, Kobe, Japan. E-mail: chad\_brick@sumibe.co.jp

...AS THE POTENTIAL OF UNLIMITED ONLINE publishing becomes clear, we

will see a greater number of publications that end in negative, unexpected, or less interesting results. From the perspective of students, who have limited time, the publication of experiments that they should avoid would be remarkable. For researchers with limited funds, it would be invaluable, and to those organizations providing funds, it would be of great commercial interest as it provides a means of ensuring they do not keep funding research that has already been carried out....

**RODDY GRIEVES**

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...IN THE FUTURE, SCIENCE WILL DICTATE policies. Governments will ask for scientific reports every time there is the need for a new

law. Voters will have extensive scientific knowledge and will make their daily life decisions based on science's latest findings.... How will science gain such importance in society? Because tomorrow's

scientists, research institutes, and universities will open their lab doors to the general public, as well as to designated political and civil representatives, on a regular basis! A visit to a lab will be just like any other way of spending a free afternoon; to many, more interesting than a museum, a park, or even retail therapy...

**ANA NEVES-COSTA**

Cell Biology of the Immune System Unit, Institute of Molecular Medicine, Lisboa, 1700-202, Portugal. E-mail: ancosta@fm.ul.pt

TECHNOLOGY WILL PUSH THE BOUNDS OF science communication in the next half-century. We'll share our results via Star Trek-like holodeck simulations: 3D interactive environments where we can walk through a molecule to see its structure, or traverse simulated forests to understand changes in biodiversity. These technologies will help the public understand science by making it real—they can feel the effects of increasing CO<sub>2</sub> on their skin, rather than just staring at a graph of yearly temperature changes. But the core of science communication won't change; our ability to explain complicated concepts using regular language and our passion for scientific discovery can only be conveyed when we talk with someone face to face....

**ALLISON COFFIN**

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...TRYING TO READ ALL THE PAPERS ON, SAY, autism or brown dwarfs 50 years from now (or even today) will be impossible.... You'd spend too much

time manually prowling databases for what is relevant.... You want truly personalized structure, integration, and filters? You want it fast? Cut out the middle man.... Individuals will each possess their own private brain Internet cache, and when a new experiment is performed, the cache will update. The way each update is processed will be unique to each individual, because plasticity—what is perceived as important enough to remodel dendrites, thus linking past with present experiences—is unique. Plus, you can buy a special filter to keep from turning into a zombie.

**JUSTIN JEE**

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## Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the past 3 months or matters of general interest. Letters are not acknowledged upon receipt. Whether published in full or in part, Letters are subject to editing for clarity and space. Letters submitted, published, or posted elsewhere, in print or online, will be disqualified. To submit a Letter, go to [www.submit2science.org](http://www.submit2science.org).



## STATISTICS

## Great Refresher or First Course

Evelyn J. Lamb

“Nixon couldn’t have won. I don’t know anyone who voted for him.” In a chapter of *Naked Statistics* subtitled “Garbage in, garbage out,” Charles Wheelan uses this apocryphal quote by the late (politically liberal) film critic Pauline Kael to illustrate selection bias. We all recognize that a survey of our friends or co-workers is not representative of Americans as a whole, but we don’t always think about the fact that scientists and pollsters must work hard to fight against the same bias when they collect data. Wheelan (an economist at Dartmouth College) follows Kael’s quip with the story of a poll, conducted by the magazine *Literary Digest* during the 1936 U.S. presidential campaign season, that predicted that

Republican Alf Landon would beat Democrat Franklin Roosevelt. The poll had been sent to an astounding 10 million subscribers to the magazine and automobile and telephone owners, who in 1936 tended to be wealthier than average Americans. The sample was huge, but it was also skewed. Wheelan comments, “As polls with good samples get larger, they get better, since the margin of error shrinks. As polls with bad samples get larger, the pile of garbage just gets bigger and smellier.”

Statistics is in the spotlight right now. As data become increasingly easy to collect and analyze, media consumers are bombarded with more and more number-based information. Nate Silver’s “victory” in the 2012 election showed that fairly simple statistical analysis of polls could far outperform political pundits at predicting the results of close races. But it also revealed how ignorant many people are of statistics. Wheelan’s accessible, entertaining book comes at an opportune time for helping the public interpret the numbers they read in the media.

*Naked Statistics* hits the typical first-year statistics topics—median, variance, the central limit theorem, regression analysis, and so on—with an emphasis on what each subject means for the average citizen. The early chapters will be a refresher course for many readers, but the following ones on data collection and regression cover ground not often seen in popular writing on the subject. Some examples are very familiar to those of us in the world of math and science communication, but they often bear repeating. The half-chapter on the Monty Hall problem offers one of the better expositions of the seeming paradox I have read. Wheelan’s examples are most

effective when he bases them on real data. I grew weary of the implausible scenarios involving broken-down buses, which sometimes sounded like the far-fetched word problems of my high school math and physics classes.

Throughout the book, Wheelan weaves a common thread of skepticism. He encourages readers to consider alternate explanations for correlations between two variables before leaping to a causal conclusion. Are test scores consistently rising

at a high school because the teachers and principal are unusually effective? Or are the worst students dropping out?

In his discussion of recall bias, Wheelan describes a study of diet and cancer risk based on women’s recollections of what their diets had been in the past. Women who had been diagnosed with cancer reported eating higher-fat diets. But as Wheelan emphasizes, it wasn’t actually a study about how diet affects cancer rates: “This was a study of how getting cancer affects a woman’s memory of her diet earlier in life.” Women with cancer had recalled eating a higher-fat diet because, consciously or not, they associated high-fat diets with a higher risk for cancer. Examples like this are part of why the book was described in the *New York Times* as “the most important health book of the year” (1). Health news is among the most common sources of statistics that most people encounter—and one in which their decisions have the weightiest consequences.

Wheelan’s book goes beyond the typical mantra of “correlation does not imply causation.” One of its most important messages is that statistics can never prove causation. They provide evidence that can be bolstered or undermined by common sense, the discovery of a mechanism, or further studies, but an article that suggests a causal relationship based on a regression analysis is overselling the science.

In a data-saturated world, statistics are often used to manipulate people. Lobbyists on both sides of hot-button issues such as gun control, genetically modified food, and federal spending use numbers to back up their claim, often cherry-picking data sets or implying a causation when there is only a correlation. A more numerate population will be less likely to fall for statistical sleights-of-hand. *Naked Statistics* can help.

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10.1126/science.1236436

**Naked Statistics**  
 Stripping the Dread  
 from the Data

by Charles Wheelan

Norton, New York, 2013.  
302 pp. \$26.95, C\$28.50.  
ISBN 9780393071955.

## BROWSINGS

**Views of the Cordilleras and Monuments of the  
 Indigenous Peoples of the Americas: A Critical Edition.**

Alexander von Humboldt. Vera M. Kutzinski and Ottmar Ette, Eds. J. Ryan Poynter, Translator. Giorleny D. Altamirano Rayo and Tobias Kraft, Annotators. University of Chicago Press, Chicago. 2012. 658 pp. \$65, £42. ISBN 9780226865065.



Humboldt does not retrace the itinerary of his 1799–1804 voyage to the Americas, nor does he organize the 69 plates and 62 commentaries along historical, cultural, or thematic lines. Instead—weaving together art, science, and his readings—he presents multifaceted perspectives on objects, ruins, and landscapes (such as *Air Volcano of Turbaco*, mud volcanoes near Cartagena, Columbia, above) that he encountered. In their introduction, the editors discuss Humboldt’s cosmopolitan stance on world cultures, including his rejection of beliefs that the New World was a continent without history or civilization.

## GENOMICS

# What If Extinction Is Not Forever?

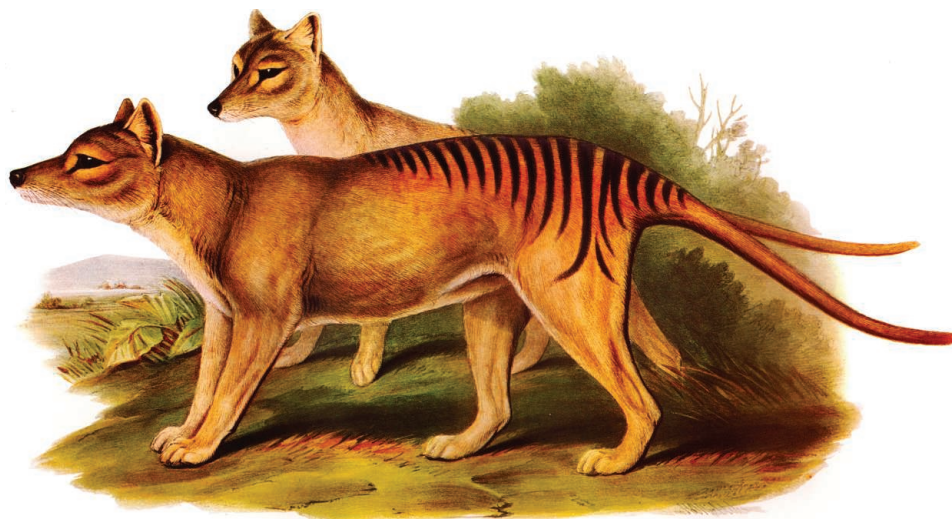
Jacob S. Sherkow<sup>1</sup> and Henry T. Greely<sup>2</sup>

A 1930s film shows a dog running and jumping inside a fenced enclosure (1)—except that the dog has a strange-shaped head, odd stripes, and a rigid tail that can only move side-to-side. The “dog” is actually one of the last thylacines, a marsupial predator also called the Tasmanian tiger. The film was taken shortly before humans extinguished the species forever. Or did we? Recently, new technologies have made it plausible to try to revive many recently extinct species. Scientists around the world are discussing, and working toward, “de-extinction” (2).

Currently, three approaches to de-extinction seem most likely to succeed: back-breeding, cloning, and genetic engineering. If the extinct species left closely related descendants, it might be possible to use selective breeding to produce progeny with the phenotypes of the extinct species, as the auroch project in Europe has been doing since 2008 (3). With newly cheap genome sequencing methods, one might guide back-breeding with genome sequences from samples of the extinct species. Of course, back-breeding will only be possible in situations where the genetic variations of the extinct species survive in the descendant species.

Cloning provides another possibility. Using cryopreserved tissue from the last known Pyrenean ibex, a Spanish group used somatic cell nuclear transfer (SCNT) to revive that extinct subspecies. Out of several hundred efforts, however, only one fetus survived to term, and it died minutes after birth from lung abnormalities (4). This example highlights two problems with SCNT: it is neither very safe nor efficient and will only work if viable cell nuclei are available. This will likely be the case in only a few very recent extinctions.

Genetic engineering offers a third approach. Take an extinct species—say, the passenger pigeon—that left sufficient samples to allow high-quality whole-genome sequencing. DNA in cells from a similar living species—perhaps the band-tailed pigeon—could be edited to match



**Tasmanian tiger.** By the 1930s, settlers, encouraged by government bounties, had hunted the thylacine to extinction in the wild. Well-preserved specimens could pave a way to bringing it back.

the extinct species' genomic sequence. The modified cells could then be used to produce living birds that, genomically, were mainly band-tailed pigeon but partially passenger pigeon (5). By using targeted replacement of genomic sequence (6) across several loci, much of the extinct genome could be reconstructed within several generations.

Neither the back-breeding nor genetic engineering approaches would yield an animal that had exactly the same genome as any member of the extinct species for many years, if ever. The cloning approach, in the few cases where viable nuclei are available, would produce a genomic twin to one member of the extinct species—but only one. Does one individual (or a set of clones) make a “species”? Even if genomic identity is necessary, is it sufficient? The revived individuals would not have the same epigenetic makeup, microbiome, environment, or even “culture” as their extinct predecessors.

## Risks and Objections

Objections to bringing back extinct animals fall into five categories: animal welfare, health, environment, political, and moral.

Animals created in the de-extinction process could end up suffering, either as a result of the processes used or because of their particular genomic variations. We know, for example, that SCNT can lead to

Although new technologies may make it possible to bring extinct species back to life, there are ethical, legal, and social ramifications to be addressed

high levels of deformity and early death (7). The Animal Welfare Act and its institutional animal care and use committees limit precisely this kind of suffering (8). Beyond physical suffering, some animal advocates might oppose de-extinction as they oppose zoos—on the grounds that they exploit animals for unimportant human purposes, like entertainment.

Newly de-extinct creatures might prove excellent vectors for pathogens. An extinct animal's genome could also conceivably harbor unrecognized, harmful endogenous retroviruses.

If the species either is released or escapes into the general environment, it might do substantial damage. Even extinct species that were not pests in their past environments could be today. For example, less than 200 years ago, billions of passenger pigeons migrated each year between the eastern United States and Canada. Today, those regions have far more humans, far larger urban centers, very different agriculture, and largely transformed ecosystems. The American chestnut, a main food source for the passenger pigeon, is now nearly extinct in the wild. Even in the same location, the passenger pigeon would today be an alien, and potentially invasive, species—perhaps another starling or even an avian kudzu.

The political risks are considerable,

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too. Current protection of endangered and threatened species owes much to the argument of irreversibility. If extinctions—particularly extinctions where tissue samples are readily available—are not forever, preservation of today's species may not seem as important. Also, genetics and, more broadly, modern bioscience, could face a backlash if citizens perceive public investments in bioscience as being used to revive species rather than cure human disease.

Finally, some people will complain that, whatever its consequences, de-extinction is just wrong—it is “playing god,” “reversing natural selection,” or an act of hubris. Others may argue that we cannot know enough about the consequences to re-introduce a species. But neither do we know the full consequences of its extinction or its continuing nonexistence.

### Benefits

Like the risks or objections to de-extinction, we see the benefits falling into five categories: scientific knowledge, technological advancement, concrete environmental benefits, justice, and “wonder.” These benefits are quite similar to the arguments made for preserving currently endangered or threatened species.

De-extinction could allow scientists the unique opportunity to study living members of previously extinct species (or, at least, close approximations to those species), providing insights into their functioning and evolution. Some revived species may be translated into useful products; for example, it is conceivable that new drugs may be derived from extinct plants.

De-extinction could lead to technological advances. The most likely would be improvements in genetic engineering, such as the targeted replacement of large stretches of genomic DNA (6).

Some researchers argue that “re-wilding” with existing species, locally extinct or threatened ecosystems (9). The same can be argued about the restoration of extinct species. The revival of the woolly mammoth as a major grazing animal in the Arctic, for example, might provide substantial benefits by helping restore an arctic steppe in the place of the less ecologically rich tundra (10).

Justice is a viscerally attractive argument for de-extinction, at least for species that humans drove to extinction: We killed them. We have the power to revive them. We have a duty to do so. But to whom or what do we owe that duty? Would it apply to all

species in whose extinction humans played the sole, the leading, or a substantial role?

The last benefit might be called “wonder,” or, more colloquially “coolness.” This may be the biggest attraction, and possibly the biggest benefit, of de-extinction. It would surely be very cool to see a living woolly mammoth. And while this is rarely viewed as a substantial benefit, much of what we do as individuals—even many aspects of science—we do because it’s “cool.”

### Legal Issues

We may also need to consider several legal issues. First, would a de-extinct species be “endangered”? The answer is unclear. In the United States, the Endangered Species Act provides for listing as “endangered” any species “over utilized” for scientific purposes, inadequately protected by current regulations, or whose existence is threatened by other “manmade factors” (11)—all considerations that would seem to apply to a newly revived species. Ironically, international organizations typically tie endangered status to whether species’ population has declined—the opposite of the concern about newly revived species (12). Uncertainty about the status of de-extinct species will affect numerous civil, criminal, and international laws.

Second, could a revived species be patented? This answer also seems unclear. The United States and many other countries allow patents on living organisms (13). Although “products of nature” cannot be patented, is a revived species a “product of nature” in light of the inevitable differences from its predecessors? Additionally, the “lost arts doctrine” may allow the patenting of previously existing species if they have been completely lost to the public (14).

Last, would de-extinction be regulated and if so, how? Again, the answer is unclear. And even if there were no legal regulation, the concerns previously discussed could dampen the enthusiasm for de-extinction by some research entities, such as universities. This could drive the efforts toward less controlled, or constrained, enterprises.

### What Should Be Done?

The answer to the question—What to do about de-extinction?—depends in part on closely defining the question. Consider three different “bottom-line” questions.

First, should de-extinction be publicly funded? This answer seems, to us, “largely no.” The potential tangible benefits from de-extinction are too small and the potential objections are too serious to justify sub-

stantial government expenditure. One might argue that governments fund science projects with similarly small practical relevance, but those “cool” projects, like the Mars rovers, present fewer risks and objections.

Second, should de-extinction be categorically banned? Here the answer seems a fairly clear “no.” The risks look fairly small and probably manageable. If people want to devote their own time, money, and efforts to the endeavor, the risks to the world do not seem to justify complete prohibition.

Third, should de-extinction be regulated? Here, we think the answer is “Yes—somewhat.” The animal welfare and environmental concerns are real. They could be mitigated by protective action but only if the law requires it. Bringing all de-extinction efforts under something like the Animal Welfare Act and requiring careful environmental assessments before any planned releases (as well as approved precautions against inadvertent release) do seem appropriate. Whether other kinds of regulation are needed is less clear, although there may be some cases, like any attempted revival of extinct hominid species, where special controls, or bans, would be appropriate.

De-extinction is a particularly intriguing application of our increasing control over life. We think it will happen. The most interesting and important question is how humanity will deal with it.

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# Dynamics of Coral Reef Recovery

Beth Polidoro<sup>1,2</sup> and Kent Carpenter<sup>1,3</sup>

**A**mong all marine habitats, coral reef ecosystems support the highest concentration of marine biodiversity. Yet, corals are declining around the world at an alarming rate, mainly as a result of more frequent, larger, and longer-lasting bleaching events observed in recent decades (1–3). The study of reef resilience and recovery at the isolated Scott Reef, reported by Gilmour *et al.* on page 69 of this issue (4), offers hope that coral reef ecosystems can recover after mass bleaching events, if anthropogenic threats can be greatly reduced or eliminated.

Coral reefs bleach when coral polyps lose their symbiotic zooxanthellae, or microalgae, which are critical for providing nutrients through photosynthesis. Coral bleaching is a response to stress, which can be caused by a number of local or regional anthropogenic disturbances, including sedimentation, pollution, destructive fishing techniques, and overexploitation of fish and other reef inhabitants that maintain optimal reef conditions and macroalgal growth. The increased duration and frequency of global mass bleaching events are also strongly linked to increased sea surface temperatures and ocean acidification associated with global climate change (5, 6). One-third of the world's 845 species of reef-building corals are considered to be at elevated risk of extinction (7) as a result of the combined effects of global climate change and local anthropogenic impacts. The most pessimistic scenarios estimate that the world's reefs may entirely disappear within this century, but other scenarios based on variable zooxanthellate thermal tolerances and reduced greenhouse gas emissions predict persistence into 2100 (5, 8).

Coral reef recovery is mainly thought to depend on recruitment or arrival of larvae from distant, interconnected reef ecosystems. Local recruitment from surviving individuals, such as those in deeper or adjacent areas, is thought to occur for various marine populations such as marine fishes (9,



**Coral reef ecosystems at risk.** Around the world, bleaching events in coral reef ecosystems are putting coral reef species at risk of extinction. Gilmour *et al.* show that isolated reefs with minimal disturbance from human activities can recover more easily from bleaching. The purple sea fan (or soft coral) in the left image is an inhabitant of coral reefs built by reef-building corals mostly in the order Scleractinia. On the right, a butterfly fish swims behind fire coral, one of the few non-Scleractinian species that contribute to reef building. Both images were taken in the Mesoamerican Reef off the coast of Roatan, Honduras.

10), but has not been well documented for coral species. Estimates of maximum dispersal distance and larval survival for reef-building coral species are sparse. However, evidence that the larvae of five reef-building coral species survive longer than expected suggests that long-distance dispersal, although rare, may be greater than currently assumed and highly variable depending on the coral species (11).

The relatively rapid recovery of coral cover following a mass bleaching event at the isolated Scott Reef documented by Gilmour *et al.* suggests that corals can recruit from local sources, especially in the absence of anthropogenic disturbances, which have been shown to slow down recovery (12). Continued anthropogenic pressure can cause changes in ecosystem dynamics such as increased algal growth from pollution and loss of herbivorous fish from overfishing or other impacts. As a result, degraded or destroyed reefs become harder to reseed from healthy, interconnected reefs

(13). Loss of live coral cover can also create negative feedbacks that inhibit ecosystem recovery. For example, loss of structural heterogeneity can cause a decline in the abundance and diversity of reef fishes, especially in isolated reef ecosystems (10, 14). A recent study in the Caribbean suggests that when the area of live coral in a reef drops below ~10%, erosion may begin to outpace the growth of new reef structures (15).

Some coral and fish species are likely to recolonize faster than others, whereas others might not recolonize at all (10). However, most studies on coral reef loss and recovery are based either on the percentage of live coral cover lost or remaining, or on the loss or recovery of selected genera or key reef species, such as the iconic Elkhorn coral (*Acropora palmata*) and Staghorn coral (*Acropora cervicornis*) in the Caribbean. It is nearly impossible to quantify all coral reef species present either before or after a mass bleaching event, especially in regions such as the Coral Triangle in the Indo-Malay

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Philippine Archipelago, where more than 500 species of reef-building corals can be found in the same area. Many coral species are very difficult to identify to the species level in the field. Additionally, further taxonomic and/or genetic studies are needed to determine the validity of a large number of reef-building coral species, especially those in the family Acroporidae (7).

The study by Gilmour *et al.* prompts us to ask how many of the world's reef systems are as isolated from anthropogenic impacts as Scott Reef, and to what degree they are benefiting from local recruitment. What will be the impact of increased ocean temperature and acidification on the long-term survival of all reefs, especially as even isolated reefs are susceptible to climate-driven reef degradation (14)? Across all coral reef ecosystems, more research is needed on which

species are more resilient to local or global threats and which species are at highest risk of little or no recovery.

Without more comprehensive species-specific information and research, it remains unclear whether coral species composition and regeneration in recovered reefs can ever reach the same state of previously healthy, undisturbed reefs. It is clear, however, that substantially reducing anthropogenic impacts on coral reefs might at least buy us, and coral reef ecosystems, more time to answer these questions.

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## NEUROSCIENCE

# A Trace of Your Place

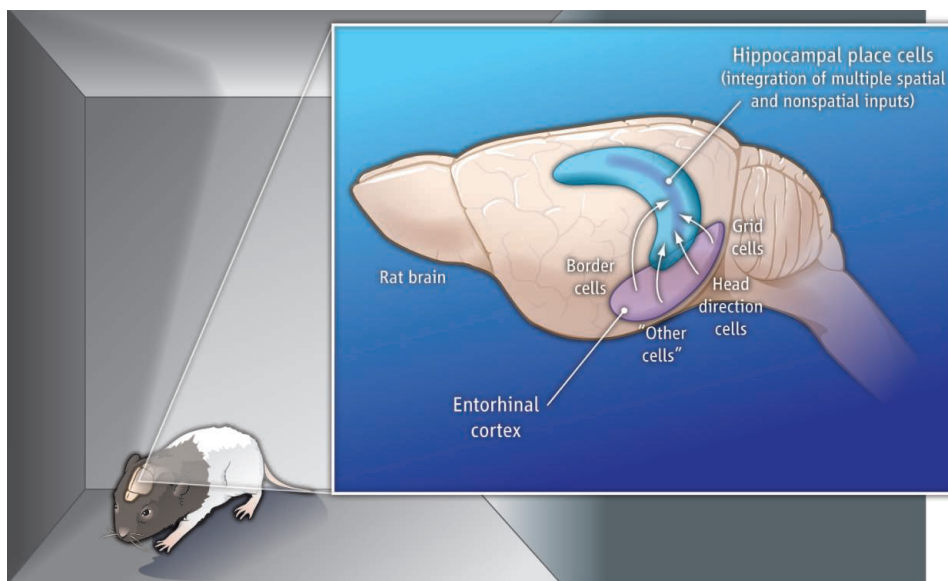
Bruno Poucet and Francesca Sargolini

**H**ow do you know where you are? How do you generate or remember a mental map of your surroundings? Spatial cognition arises from multiple neural systems in the brain that converge to provide this awareness. Teasing out the origin of brain signals that course through these networks is tremendously difficult because the number and nature of pathways in the brain that give rise to a particular signal—the afferent inputs—are often not known. This becomes even more complicated if one looks for connectivity along these pathways at the level of single neurons, the most elementary operating units in the brain, because of the thousands of connections a neuron can make with other neurons. On page 44 of this issue, Zhang *et al.* (1) tackle this question by identifying the afferent cells in the entorhinal cortex—a region associated with navigation and memory—that send signals to the hippocampus, specifically to “place cells,” neurons that become active in response to a specific location in an environment. The scheme that emerges establishes functional connectivity at an unprecedented level of detail.

The study of spatial cognition has benefited from experimental paradigms that can be made relevant to humans or animals, such as rodents. It has been known for more than 40 years that when rats freely move in a small

Multiple signals of different cellular origin in the brain's cortex must be integrated in a single cell type in the hippocampus to assess spatial location.

arena, pyramidal cells (a type of hippocampal neuron) show a peculiar activity—certain cells “fire” (send an electrical signal) when the animal is in a specific area. These pyramidal cells are “place cells” (2), and the ani-



**Mental map of location.** The hippocampus in both humans and rats is involved in spatial awareness and navigation. All cell types in the medial entorhinal cortex (rat shown) project to place cells in the hippocampus. These inputs include cells with strong spatial correlates (grid cells, border cells, and head direction cells), but also cells with much weaker or no spatial signal. How these signals are all integrated to give rise to the place cell signal remains an unresolved issue.

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mal's particular location in a particular environment that activates specific place cells is called the "place field." There does not appear to be a topographical relationship between the anatomical location of the cells within the hippocampus and the place fields of these cells in an environment. Multiple cues are thought to contribute to a place cell's activity such as external landmarks and the animal's own motion (3). However, the underlying neural circuitry responsible for generating a place field is still a matter of debate. Because the vast majority of cortical inputs to the hippocampus stem from the entorhinal cortex, it is plausible that information about both external cues and self-motion is relayed by entorhinal neurons. This hypothesis has received strong support from the discovery of grid cells (4) in the medial entorhinal cortex. Grid cells resemble place cells in that they display location-specific firing and process both external landmarks and self-motion cues (5). However, in contrast to hippocampal place cells (which usually have a single place field in a given environment), grid cells fire at multiple locations in a very regular grid-like pattern. Given that grid cells directly project to the hippocampus, it is tempting to hypothesize that place cell signals may result from the convergent input from several grid cells, with the integration of grid cell activity by place cells obeying sophisticated transformation rules. More recent research, however, has revealed that the medial entorhinal cortex also contains cells that code the animal's head direction (6) or the presence of borders in the environment, as well as other cell types that carry a weak

spatial signal or no spatial signal at all (7). All of these different types of cells could potentially influence place cell activity or even contribute to the generation of their location-specific signals.

To characterize the precise functional anatomical connections between the entorhinal cortex and hippocampal place cells, Zhang *et al.* combined optogenetic activation of single neurons and electrophysiological recording of the same neurons in freely moving rats. A recombinant adeno-associated virus was engineered to deliver a gene encoding an ion channel that is sensitive to light. When injected into the hippocampus of the animal, the viral vector was taken up by the axons of the neurons in the entorhinal cortex that project to the hippocampus. When stimulated by light, these neurons could be activated and therefore be identified according to their electrophysiological signature. Parallel recordings of the same projecting neurons were taken while the animal freely explored an arena, allowing measurement of the spatial information carried in these neurons. Thus, this method enables characterization of the cortical signals that give rise to the activity of hippocampal place cells.

Surprisingly, Zhang *et al.* report that all cell types in the medial entorhinal cortex were tagged with approximately equal proportions by this technique, indicating that place cell activity results from convergent input signals from several functional cell types. Thus, it is unlikely that place fields result from a simple summation of several converging grid cell fields or any other spe-

cific signal from the medial entorhinal cortex. This makes sense, given the major properties of place cells. For example, place cells usually discharge regardless of the animal's head direction in open environments, and their activity is controlled by visual cues but remains unchanged in total darkness. These features reflect the highly integrative nature of their signal, which is possible only if they receive a wide variety of inputs.

The findings raise numerous questions about how place cells combine the different signals to generate a reliable place field. Perhaps place cells receive different inputs from the medial entorhinal cortex. It will also be interesting to determine whether there are different categories of place cells according to the type of input they preferentially gate. The answers should guide our understanding of the origin of the place-selective signal of place cells, which remains an open question, and help us to understand how humans, whose hippocampus also contains place cells (8), form a mental map of the environment.

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## PHYSIOLOGY

# The Perfect Hypnotic?

Emmanuel Mignot

The market for hypnotics is big business, with 10 to 15% of the population in the United States suffering from chronic insomnia (1, 2). Although the pathology is unknown, and likely heterogeneous, patients with insomnia are in a state of hyperarousal (even during the day), suggesting that wake-promoting systems are hyperactive (3). The search for the ideal hypnotic has been marked by cycles of exuberance followed by disappointment, as adverse side effects associated with each new class of drug

have emerged following wide use. Although insomnia therapy increasingly uses cognitive behavioral therapy, the enormous number of sufferers mandates new pharmacological approaches. Uslaner *et al.* (4) report the prospect of a new class of compound with a new mode of action that may usher in a new era for insomnia treatment, with the potential for fewer side effects.

At the beginning of the 19th century, chloral hydrate, meprobamate, and barbiturates were touted as nonaddictive miracle tranquilizers, but in the 1950s, their potential for tolerance and severe addiction was recognized (5). At high dose, they lead to pulmonary

arrest and death, outcomes that gained further notoriety with the deaths of celebrities Marilyn Monroe and Jimi Hendrix. Broad inhibition of brain activity through high-dose barbiturates can even induce a "flat" electroencephalogram mimicking brain death. Barbiturates activate a chloride channel receptor for gamma-aminobutyric acid [type A (GABA<sub>A</sub>)]. GABA is the main inhibitory neurotransmitter, produced by 15 to 20% of all brain neurons.

Hope for a safe, effective hypnotic reemerged with chlordiazepoxide, the first benzodiazepine [(BDZ); a two-benzene ring structure linked by a third, diazepine ring].

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A compound that blocks a neurotransmitter receptor is the newest hope for getting a good night's sleep without the bad side effects.



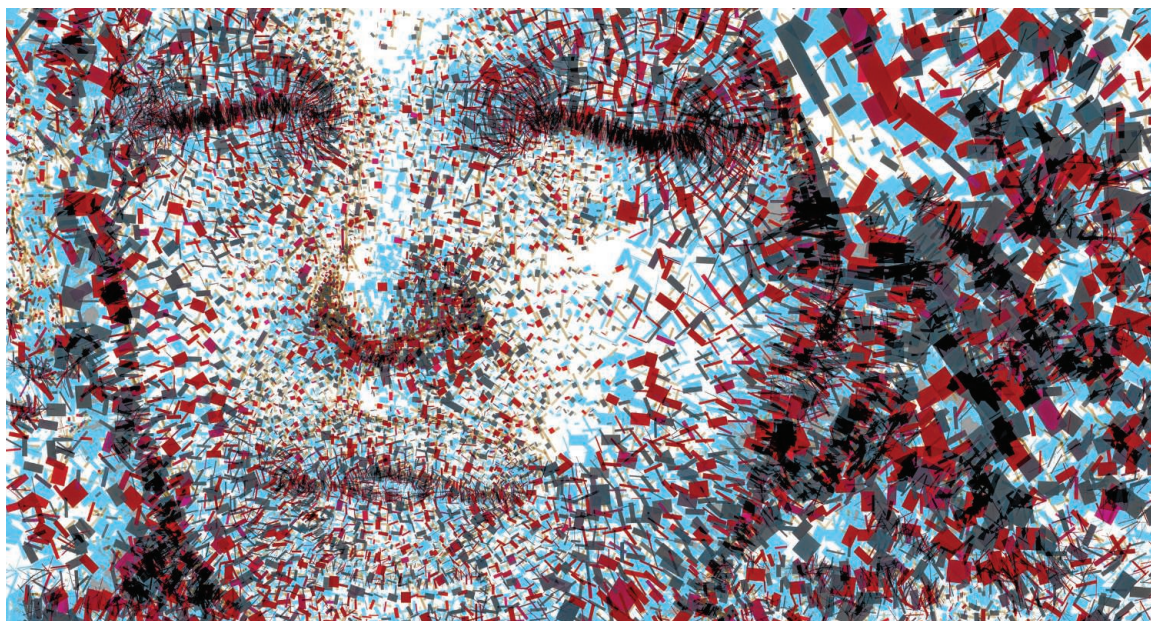
BDZs are safer, rarely causing respiratory depression, even when “overdosed,” and more than 40 derivatives (including the prototypical diazepam) have been synthesized. Prescribed variously for sleep induction or maintenance, as anticonvulsants, muscle relaxants, or as antianxiety (anxiolytic) agents, these drugs were proclaimed as the perfect solution. But they too fell out of favor when long-acting compounds were found to induce residual sedation, memory loss, and addiction. BDZs bind to the GABA<sub>A</sub> receptor at a site that is different from that for barbiturates, yielding similar but less extensive effects (5, 6). The residual effects, however, spurred development of short-half-life hypnotics in the 1980s (e.g., triazolam). Although effective, these compounds manifested other problems: rebound insomnia on cessation and occasionally a state of “confusional arousal” in which the patient may be acting out unaware, half asleep but moving around, not unlike sleep-walking (this led to legal cases). Triazolam was withdrawn from many markets and/or the dose decreased.

Then came the “Z-drugs”—zaleplon, zolpidem, and zopiclone (and eszopiclone)—short-half-life compounds with lower effective doses and improved side-effect profiles. Although lacking the three-ring BDZ structure, they recognize the BDZ-binding site of the GABA<sub>A</sub> receptor and manifest receptor subtype specificity such that some have primarily hypnotic (zolpidem) rather than both hypnotic and anxiolytic (eszopiclone) effects (6). Although safer than classic BDZs, occasional problems with dependence, tolerance, and “confusional arousals” are still reported with Z-drugs in at-risk populations. These BDZ-like compounds are now the “gold standard” treatment for insomnia, although they are “scheduled” substances by the U.S. Food and Drug Administration (their use and distribution are tightly controlled because of abuse potential) and viewed with some suspicion by doctors and patients.

In parallel with this drug evolution, sedatives (e.g., chlorpromazine) were introduced to treat agitation in psychotic patients in the 1950s. These compounds block receptors for the neurotransmitter dopamine, although sedative effects mostly correlate with their ability to block H1 histaminergic receptors that relay wake-promoting histamine signals within the

brain. Antihistamines were developed to treat allergies but soon found utility as remedies for insomnia. More “natural” sedatives, such as the hormone melatonin, have a modest hypnotic effect, typically insufficient for severe insomnia, although typical doses far exceed normal physiological amounts. Melatonin or melatonin agonists (such as ramelteon) activate cognate receptors in the brain to provide a “darkness” signal that may be useful, when combined with light therapy, to adjust

of diazepam (a BDZ hypnotic), and eszopiclone and zolpidem (BDZ-like hypnotics) in rats and monkeys and concluded that DORA-22 promotes sleep at doses that do not impair cognition and memory, in contrast to BDZ and BDZ-like compounds. The experimental task studied included novel object recognition by rats, with associated changes in hippocampal Arc expression, a measure of synaptic plasticity believed to correlate with memory retention. These measures were all



**On the horizon?** A compound that blocks the wake-promoting effects of orexin hormones may usher in a new era for insomnia treatment.

circadian rhythms. Similarly, wary of addiction to GABAergic drugs, many patients are prescribed sedative antidepressants that block the H1 histamine or the 5-hydroxytryptamine (5HT<sub>2</sub>) serotonin receptors. These compounds were never developed for insomnia, and have long half-lives, thus causing residual daytime sedation (5).

Uslaner *et al.* (4) explored the effects of a new class of hypnotic compounds that are antagonists of the two receptors for orexin, so-called dual orexin receptor antagonists (DORAs). Orexins (also called hypocretins) are key neurotransmitters of arousal in the central nervous system. Genetic and pharmacological studies pointed to modulation of the orexin system as potential therapy for insomnia and other sleep disorders (7, 8). DORAs have fewer effects on daytime performance tests, and no rebound insomnia on cessation (9), although there are residual dose-dependent sedative effects (10), and high doses administered during the day can impair human performance. Uslaner *et al.* (4) compared the effects of DORA-22 with those

impaired by diazepam, zolpidem, and eszopiclone but not by DORA-22. Similarly, the ability of monkeys to remember and match colors after a small delay (a working memory task) or to react rapidly to the appearance of an object on a screen (a measure of attention) was not impaired by DORA-22, whereas all GABAergic treatments had substantial detrimental effects. What is needed next are parallel studies in humans to demonstrate generalization to a clinical population.

Unlike the broad inhibitory effects of GABA on brain activity, orexins produce selective wake-promoting signals. Although orexins are produced by only 70,000 neurons in the hypothalamus, these send widespread anatomical projections, thereby exciting other wake-promoting systems such as the histaminergic tuberomammillary nucleus, the adrenergic locus coeruleus, and various cholinergic and aminergic cell groups. Blocking orexin may thus be closer to treating the underlying issue of excess alertness in insomnia compared to promoting sleep by inhibiting brain activity.

Discovered in 1998 (11, 12), the role of orexins (there are two types, A and B, processed from the same pre-propeptide) in sleep emerged when it was discovered that impaired orexin signaling results in the sleep disorder narcolepsy in animal models and humans (7). Narcolepsy is characterized by sleepiness and abnormal rapid eye movement (REM) sleep, so that patients not only fall asleep easily but also experience symptoms where REM sleep intermingles with wakefulness, resulting in dream-like hallucinations or episodes of muscle paralysis when awake (sleep paralysis and cataplexy). Narcolepsy is caused by an autoimmune attack against neurons that express orexin.

Orexins act on G protein-coupled receptors called hypocretin receptor 1 and 2 (also called OX1R and OX2R, respectively). Although OX2R is more prominently involved in narcolepsy, both receptors may regulate sleep, although this could be species dependent. The potential for orexin antagonists to increase REM sleep and dreaming, or even narcolepsy-like symptoms, has been

a concern (8), but has not been observed to a substantial degree in clinical trials with DORA molecules (13). A small but significant increase in REM sleep and decreased REM latency were noted, and sleep may be less consolidated than after GABAergic hypnotics (9, 10, 13). As the beneficial effects of REM versus non-REM sleep on restoration of wakefulness, memory, synaptic plasticity, and mood are highly debated (14, 15), it is uncertain whether this difference in REM versus non-REM profile may be beneficial.

Are DORAs the perfect hypnotics? Only long-term use in large numbers of insomnia patients will reveal whether these drugs will be preferred to GABAergic hypnotics, and whether they produce rare complications, including narcolepsy-like symptoms in predisposed individuals (16). Side effects are expected with any active treatment, and the benefit-risk ratio must be considered. There is minimal tolerance for side effects and risk in the treatment of insomnia, so distinct drugs offering multiple modes of action,

especially complementary ones, will greatly benefit patients.

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## MICROBIOLOGY

# Breathing Perchlorate

Robert Nerenberg

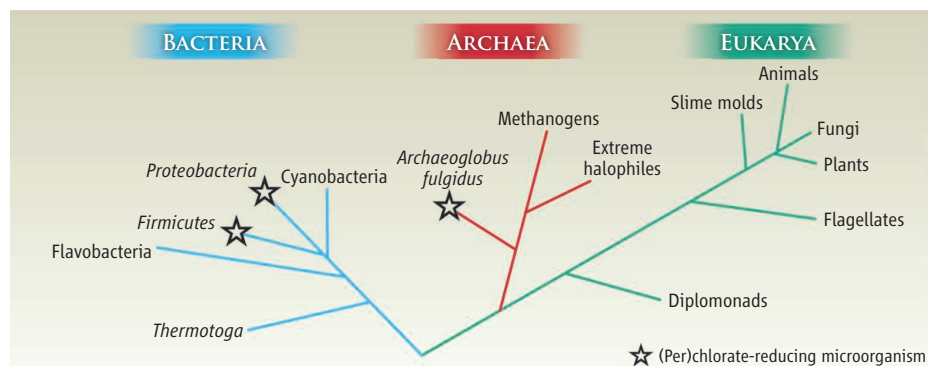
About 20 years ago, investigators discovered that perchlorate ( $\text{ClO}_4^-$ ), a synthetically produced chemical widely used in rocket propellants and explosives, was present at trace levels in many water supplies across the United States (1). Perchlorate can inhibit thyroid function, potentially leading to developmental problems in fetuses and infants (2). The public outcry led to intense research on perchlorate and the environment. Biological reduction was considered a promising treatment strategy for perchlorate, as well as for a related oxyanion, chlorate ( $\text{ClO}_3^-$ ) (1, 3). This approach is based on microorganisms that can respire on, or gain energy from, the reduction of these compounds to chloride. On page 85 of this issue, Liebensteiner *et al.* show that the hyperthermophilic archaeon *Archaeoglobus fulgidus* can also respire with perchlorate and chlorate (4). This discovery changes several paradigms about perchlorate and chlorate-reducing microorganisms.

Early studies on the microbial reduction of perchlorate and chlorate showed that denitrifying bacteria could reduce perchlorate and chlorate with the nonspecific nitrate reductase enzyme (5). However, the end product was chlorite, a reactive and toxic intermediate. This explained why denitrifying bacteria reduced perchlorate for a limited time and without growth. Other bacteria were found to have a dedicated pathway for perchlorate

An archaeon with an unusual perchlorate-reducing metabolism raises questions about the diversity and evolution of perchlorate-reducing microorganisms.

and chlorate reduction that allowed continued and vigorous growth (6). In this case, the end product was nontoxic chloride.

(Per)chlorate-reducing bacteria (the collective name for bacteria that reduce perchlorate and chlorate) prefer to respire on oxygen, if present; most can also grow via reduction of nitrate ( $\text{NO}_3^-$ ), or denitrification. Their abundance in pristine soil and water environments was explained when it



**Diversity of (per)chlorate reducers.** Phylogenetic tree showing the positions of known (per)chlorate-reducing microorganisms. Liebensteiner *et al.*'s discovery of a (per)chlorate-reducing archaeon expands the diversity of these microorganisms to a second domain of life.

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was found that perchlorate and chlorate can form from chloride by natural oxidation processes and are widely found at trace concentrations (7, 8). Perchlorate and chlorate concentrations in the natural environment may be kept low by microbial reduction.

The current paradigm is that the perchlorate reduction pathway relies on two specialized enzymes. The (per)chlorate reductase reduces perchlorate to chlorate, and also chlorate to chlorite. The chlorite dismutase transforms chlorite into oxygen and chloride (7). The latter is an extremely fast enzyme that protects the organism from chlorite toxicity. Furthermore, chlorite dismutation is one of the few biological transformations that result in the formation of molecular oxygen (9). In addition to detoxification, the produced oxygen provides additional energy.

Based on their genetic composition, all living organisms can be classified as members of one of three domains: Bacteria, Archaea, or Eukarya. Until 2008, all known (per)chlorate-reducing microorganisms were part of the bacterial domain, within the phylum *Proteobacteria*. In 2008, researchers reported the first (per)chlorate-reducing bacterium in a different bacterial phylum, the *Firmicutes* (10). Some nitrifying bacteria in the *Proteobacteria* and *Nitrospirae* phyla, as well as some cyanobacteria and some members of the Archaea, have a chlorite dismutase but no (per)chlorate reductase and cannot grow on perchlorate or chlorate (3, 11). Thus, known (per)chlorate-reducing microorganisms have been confined to two phyla in the bacterial domain (see the figure).

Liebensteiner *et al.*'s discovery that *A. fulgidus*—a previously isolated hyperthermophilic archaeon—can reduce perchlorate and chlorate expands the diversity of known (per)chlorate reducers to the archaeal domain (see the figure). In contrast to the known (per)chlorate reducing bacteria, *A. fulgidus* cannot respire on oxygen and is a hyperthermophile. It is also one of the few microorganisms known to reduce both sulfate and perchlorate.

There is no evidence that *A. fulgidus* has a chlorite dismutase gene or enzyme, and no sign of chlorite dismutase activity. Thus, at first glance the organism appears to have no protection from chlorite toxicity. However, several chemical reductants can detoxify chlorite abiotically (12). Liebensteiner *et al.* propose that the chlorite is detoxified through abiotic reduction with sulfide. Sulfide may be present in the environment, but can also be produced by *A. fulgidus* through sulfate reduction. This would provide an

endogenous, abiotic mechanism for chlorite detoxification.

The results support previous studies suggesting that the chlorite dismutase evolved separately from the (per)chlorate reductase and may have been transferred from one species to another. The discovery of a perchlorate-reducing archaeon suggests that there may be other (per)chlorate reducers in this domain. Also, other (per)chlorate-reducing microorganisms lacking a chlorite dismutase enzyme may exist in the archaeal and bacteria domains. Isolation of such species may require addition of a chlorite-scavenging compound.

Liebensteiner *et al.* show that the growth yields for *A. fulgidus* on perchlorate and sulfate are similar, despite the fact that significant free energy is available in perchlorate compared to sulfate. Why would the organism use perchlorate if sulfate is available? The concurrent use of low levels of perchlorate along with nitrate provides a metabolic advantage over bacteria that can only respire on nitrate (13). A similar effect may exist for perchlorate and sulfate.

Given that perchlorate and chlorate probably existed on Earth in early geologi-

cal times, the authors hypothesize that the capacity to respire on perchlorate and chlorate may have developed very early in the evolution of life. This metabolic capability would have developed prior to the development of photosynthesis and the development of respiration on oxygen, providing new insights into how life may have adapted to more oxidizing conditions.

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## DEVELOPMENTAL BIOLOGY

# Programmed Cell Death in Neuronal Development

Martijn P. J. Dekkers and Yves-Alain Barde

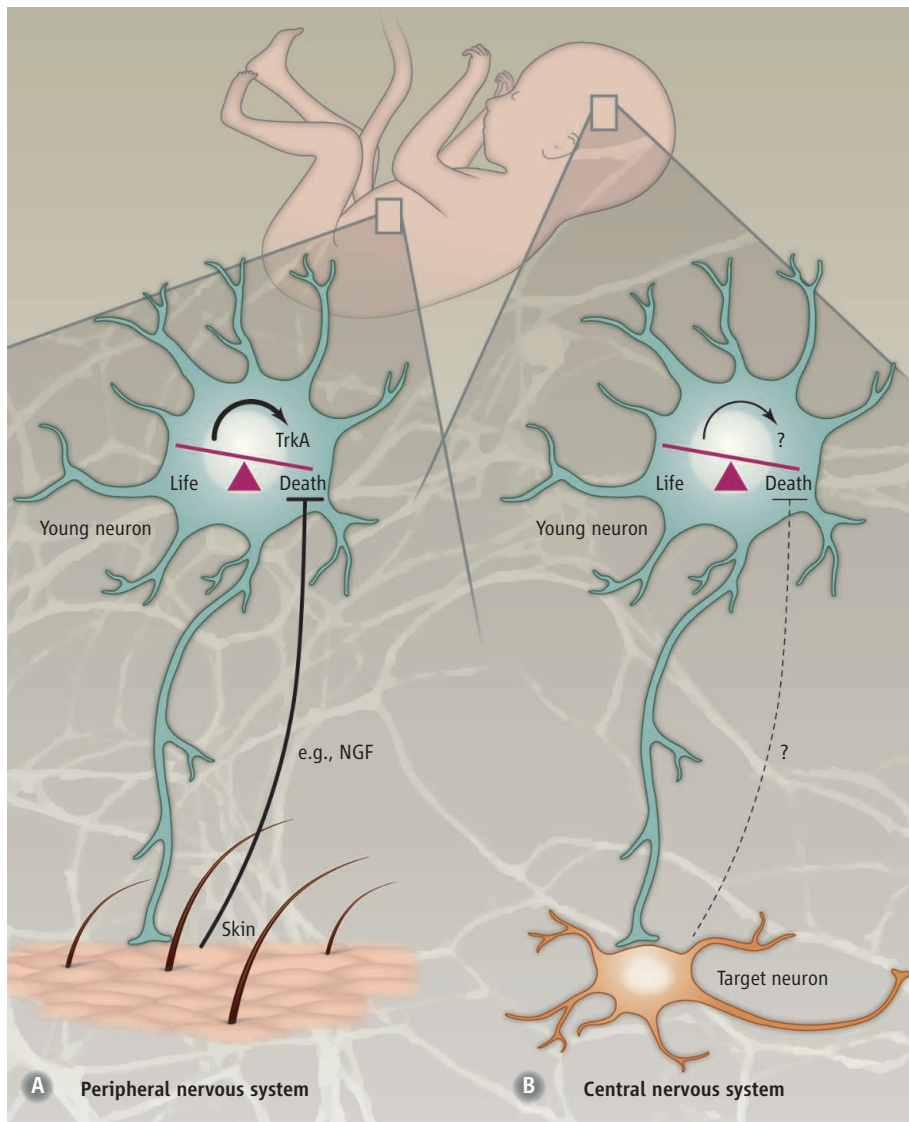
Life or death decisions in cortical interneurons during development are made independently of external cues, challenging current theory.

**P**rogrammed cell death, or apoptosis, accompanies the development of many tissues, including the vertebrate nervous system. Most neurons are eliminated soon after synaptic contacts have been made between the neurons and their targets. This inspired the neurotrophic theory, which proposes that neurons compete for limited quantities of target-derived survival factors (1–3). Work on nerve growth factor (NGF) in the peripheral nervous system (PNS) (4) gave strong support for this theory: Not only is NGF essential for the survival of specific populations of neurons, but it is also localized in tissues innervated by NGF-respon-

sive neurons in amounts that parallel the density of innervation (5). However, the finding by Southwell *et al.* (6) that programmed cell death in a major population of neurons in the central nervous system (CNS) is caused by an intrinsic program independent of external cues cannot be readily accommodated by the neurotrophic theory.

The neocortex of higher vertebrates is composed of excitatory projection neurons and inhibitory interneurons. From early in development, the ratio between excitatory and inhibitory neurons is four to one in the various subdivisions of the cortex (7). As this particular ratio is thought to be critical for brain function, cell numbers of both populations are expected to be somehow matched. During synaptogenesis, a sizable

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**Programmed cell death in the peripheral and central nervous system.** In both the PNS and CNS, programmed cell death occurs predominantly when the young neurons contact their designated target tissues (e.g., skin, or target neuron). However, the regulation of apoptosis is different. (A) In the PNS, apoptosis of the developing neurons is prevented by survival factors secreted by the target tissues (blocking arrow), which counteract the internal propensity to die (curved arrow). (B) By contrast, in the CNS, target tissue-derived signals do not seem to play a critical role in preventing neuronal cell death (dotted arrow). Rather, the decision to die or not is primarily determined intrinsically (curved arrow). The signals involved in regulating programmed cell death in the CNS are not well understood.

proportion of both excitatory and inhibitory neurons are eliminated through apoptosis (8). Southwell *et al.* found that ~40% of the interneurons in the cortex perish around this time (6), confirming earlier findings (8). Surprisingly, whether grown *in vivo*, *in vitro*, or after heterochronic transplantation, the cells underwent programmed cell death roughly 2 weeks after being generated. As both the time point of cell death and the fraction of cells affected were independent of the environment, apoptosis appears to be determined intrinsically (6). To further investigate the role of resident cells in the

developing cortex in modulating the survival of interneurons, varying numbers of interneuron precursors were transplanted in the cortex. This maneuver resulted in a limited increase in spontaneous inhibitory activity in the cortex, indicating that only a constrained number of additional synaptic contacts can be accommodated. Nevertheless, survival rates of the transplanted cells were similar for implant sizes differing up to 200-fold (6), demonstrating that neither the cellular context nor the limited possibility of functionally integrating into a network affected survival. Further, deletion of

the central apoptotic regulator Bcl-2-associated X (Bax) completely abolished the programmed (cell) death of the interneurons, and resulted in a higher number of apparently normal interneurons (6).

The dependence on extrinsic factors for neuronal survival has long been thought to be an attribute of complex nervous systems and a component of adaptive plasticity. This makes the Southwell *et al.* finding (6) all the more unexpected, and begs the question, what causes the death of cortical interneurons. One possibility is that the apoptotic cells are predisposed to die. With about half of the cortical interneurons being eliminated (6, 8), it is conceivable that the terminal division of the interneuron progenitors gives rise to a pair of cells that have a different propensity to initiate apoptosis. In the worm *Caenorhabditis elegans*, asymmetry in cell division has been linked with programmed cell death of one of the daughter cells (9, 10). The deletion of some of the genes involved in the asymmetric division of the neurosecretory motor neuron (NSM) neuroblast prevents programmed cell death of the NSM neuron (10). Alternatively, programmed cell death may be determined stochastically in cortical interneurons. In the adult germ line of *C. elegans*, ~50% of oocytes are eliminated shortly before oogenesis through a stochastic selection mechanism, possibly to rid the gonad of excess oocytes that served as nurse cells (11). The likelihood that the oocytes will initiate programmed cell death during this period is determined by several intrinsic pathways that converge on modulating transcription of the core apoptotic genes (11, 12). Similarly, the synchronous death of the inhibitory neurons 2 weeks after their generation could indicate that apoptosis is more likely to be induced during this period.

The expression of genes early in post-mitotic neurons can dramatically alter the probability that these young neurons will be eliminated. One of these is the gene that encodes the NGF receptor tropomyosin receptor kinase A (TrkA), whose expression creates a dependency on NGF for the survival of newborn neurons (13). The discovery that TrkA acts as a dependence receptor provided a long-awaited plausible explanation for why large numbers of neurons in the PNS die in the absence of NGF (13). Surprisingly, the closely related receptor TrkB, the receptor for brain-derived neurotrophic factor (BDNF), does not create a similar dependency (13), explaining why BDNF is not required for the survival of most CNS neurons (14). In line with this, the presence



or absence of TrkB does not affect survival of cortical interneurons (6). Understanding the determinants of apoptosis in these cells will require investigation of whether the interneuron precursors feature a molecular signature that is predictive for their survival or death.

Another unresolved issue is how the ratio of excitatory to inhibitory neurons is established in the neocortex. Because the number of inhibitory cells in the neocortex does not appear to be regulated through local adaptive mechanisms (6), the ratio could be determined by modulating the survival of excitatory neurons. Although many projection neurons undergo apoptosis around the time of target innervation (8), the extrinsic signals involved are still unclear. The most likely survival factor candidates have all been eliminated by gene targeting with

unspectacular results in terms of increasing normally occurring neuronal death. This is unlike what has been observed in the PNS with various growth factors, including NGF (see the figure).

The results of Southwell *et al.* also serve as a reminder of early findings that did not readily fit the neurotrophic theory. In particular, experiments in the late 1970s by Alan Lamb showed that the death of motoneurons in the spinal cord of frogs with bilaterally innervated single limbs was much lower than would have been predicted had they been required to compete for limited quantities of neurotrophic factors (1). The discovery that programmed cell death in cortical interneurons is determined intrinsically suggests anew that the neurotrophic theory should be reexamined in the context of the developing CNS.

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## CHEMISTRY

# Revealing the Positive Side of Fluorine

Ulrich Hennecke

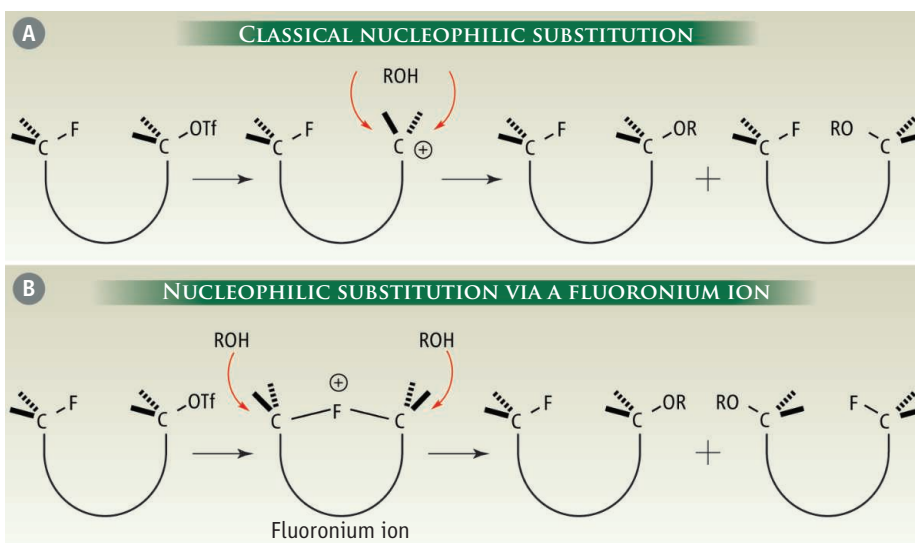
Organofluorine compounds have found widespread applications in diverse areas such as polymers, liquid crystals, and agricultural and medicinal chemistry (1, 2). Partial or full fluorination provides distinctive physicochemical properties to an organic molecule that can be attributed to the special properties of the carbon-fluorine (C-F) bond (3). Fluorine is the most electronegative (electron-attracting) element of the periodic table, so the C-F bond is highly polarized. The small size and high electronegativity of the F atom gives it a low polarizability. This property makes it difficult for fluorine's electron pairs to engage in hypervalent bonding, as other halogens can do, to form a fluoronium ion, a structure in which the fluorine is bonded to two carbon atoms with a (at least formal) positive charge at fluorine. However, on page 57 of this issue, Struble *et al.* (4) report strong evidence in support of precisely such a fluoronium ion.

Symmetrical, in many cases cyclic, halonium ions formed by chlorine, bromine, and iodine are important synthetic intermediates and have been critical in the development of

organic stereochemistry and so-called neighboring group effects. The existence of the corresponding organic fluoronium ions has always been doubted. Fluoronium ions have

An organic reaction creates an intermediate in which a fluorine atom, the most electronegative of the elements, formally bears a positive charge.

been observed only under special conditions, either in the gas phase for carbon substituents (5) or with highly reactive noncarbon substituents in solution (6, 7).



**Building a positive fluorine bridge.** Although halogens normally bear a negative charge, chlorine, bromine, and iodine can form structures in which they bear a (formal) positive charge by bridging two organic groups. Struble *et al.* now show evidence for such a structure, a symmetrical fluoronium ion, derived from fluorine, the most electronegative element. The fluoronium ion is generated in a solvolysis experiment from a precursor molecule. The outcomes of nucleophilic substitutions are shown for (A) the classical S<sub>N</sub>1 case via a carbenium ion (at the carbon atom) generating two stereoisomers, and (B) via the fluoronium ion leading to two regioisomers by direct and remote substitution.

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Struble *et al.* obtained convincing evidence for an organic fluoronium ion in solution by preparing a special polycyclic, cage-like precursor compound that provides steric protection to a fluoronium ion and limits possible decomposition and rearrangement pathways. To generate the fluoronium ion from this precursor, they used so-called solvolysis experiments, in which the precursor compound containing a leaving group (here a triflate group, -OTf) is heated in a suitable solvent (water or alcohols). Under these conditions, the leaving group is cleaved, which generates a short-lived cationic intermediate that is subsequently trapped by the solvent.

The analysis of the reaction products provides strong evidence for the intermediate generation of a fluoronium ion. In textbook-like nucleophilic substitution reactions, the substitution of the leaving group by the solvent should only occur at the carbon atom bearing the leaving group (by an  $S_N1$  or  $S_N2$  reaction) (see the figure, panel A). However, Struble *et al.* used isotopic labeling to show that substitution also occurs at the remote carbon where the fluorine was originally placed (panel B). Interestingly, direct and remote substitution is observed precisely in a one-to-one ratio, strongly indicative of a symmetrical, positively charged fluoronium intermediate.

The symmetrical structure of the fluoronium ion is further substantiated by high-

level quantum-chemical calculation supporting a fluoronium structure as the actual reaction intermediate. In the structural formula, the charge is drawn at the fluorine (because of its hypervalent connection to two substituents), placing the positive charge at the most electronegative atom. However, the calculations reveal that the charge is largely located at the two carbon atoms next to the fluorine, resolving this contradiction.

The evidence provided by Struble *et al.* contradicts the current paradigm that organic fluoronium ions are too unstable to exist. Nevertheless, the fluoronium ion presented is only a short-lived reaction intermediate attested by indirect evidence. The direct observation of a fluoronium ion through spectroscopic techniques or x-ray crystallography is still pending and will most likely require the synthesis and isolation of more stable derivatives. The current report shows that this task should be possible if the fluoronium is embedded in a well-designed cage-like structure.

Although the fluoronium was only observed as a short-lived reaction intermediate, its observation could affect synthetic organofluorine chemistry. The formation of cyclic halonium ions as reaction intermediates is well attested for the other halogens and has had a lasting effect on the stereochemistry of halogenation reactions. Struble *et al.* show that, depending on the circumstances, formation of similar ions is also

possible for organofluorine compounds. The use of tamed fluoronium ions as intermediates might enable new synthetic fluorination pathways for the synthesis of valuable organofluorine compounds.

Struble *et al.*'s results might also have wider implications in organofluorine chemistry. Because of its small size and its high electronegativity, fluorine's electrons are poorly polarizable, and organofluorine compounds generally interact with other atoms or molecules only through rather weak electrostatic interactions (3, 8). It is usually assumed that more covalent interactions like the hypervalent bonding in the fluoronium generally do not occur, but taking into account these new results, this assumption might not be general.

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## ASTRONOMY

# Modeling the Solar Dynamo

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The Sun's magnetic field is the engine and energy channel underlying virtually all manifestations of solar activity. Its evolution takes place on a wide range of spatial and temporal scales, including a prominent 11-year cycle of successive polarity reversals over the entire star. This magnetic cycle in turn modulates the physical properties of the plasma flowing away from the Sun into interplanetary space, the frequency of all geoeffective eruptive phenomena (such as flares and coronal mass ejections), and the solar radiative flux over the full range of the electromagnetic spec-

trum—from x-rays through ultraviolet, visible, and infrared light, all the way down to radio frequencies (1). The Sun's heartbeat is truly magnetic, and recent numerical simulations (2–5) are providing new insights into its mode of operation.

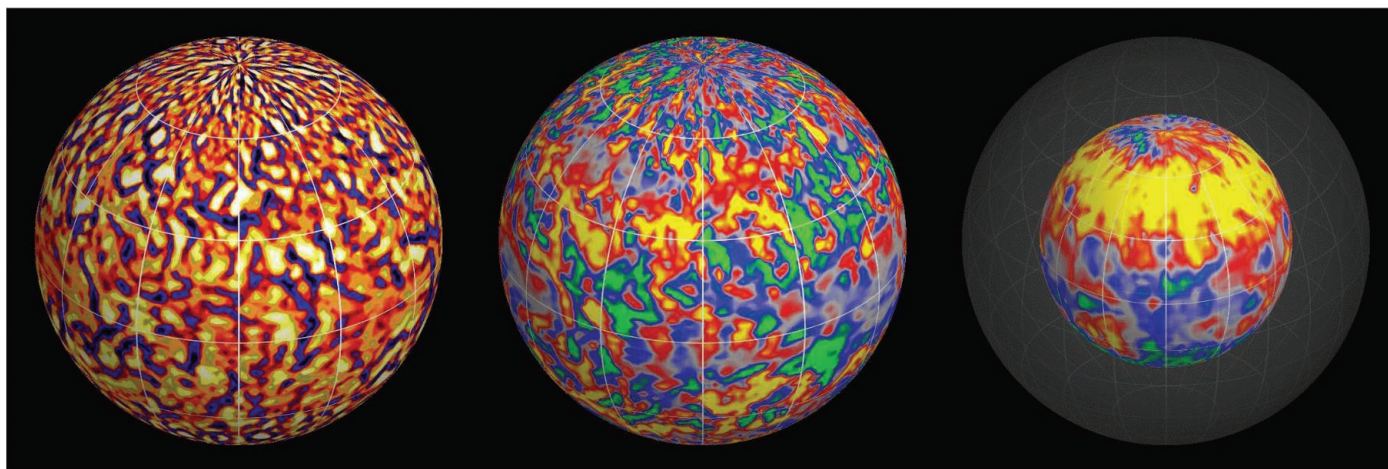
Self-sustained amplification of a magnetic field through the action of fluid motions is called a dynamo. Dynamos operate through a physical effect called electromagnetic induction, discovered by Faraday in the 19th century. Induction is put to work in modern power-generating plants converting mechanical energy imparted to turbines (by water, wind, or steam) into electricity. There are no turbines inside the Sun, but in its outer third in radial extent, the so-called convection zone, mechanical energy abounds in the form of rotational shear and

turbulent fluid motions driven by the solar luminosity. Plasma flowing across the magnetic field that pervades the solar interior induces electrical currents, which, under appropriate flow and magnetic field configurations, can sustain the field against dissipation. The magnetic field so generated in the solar interior subsequently emerges at the Sun's surface, structuring and energizing its extended atmosphere.

Parallel advances in raw computing power and ever more sophisticated numerical algorithms make it possible to produce and investigate magnetic cycles in Sun-like spheres of thermally convecting magnetized fluid. The underlying physics is in principle well understood in the form of magnetohydrodynamics (or MHD), being described by the classical fluid equations augmented by

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**Solar simulations.** Three snapshots of a magnetohydrodynamical numerical simulation of solar convection, carried out using the multiscale flow simulation model EULAG (12–15). The left panel shows a color rendering of the radial component of the convective flow (orange to light yellow, upflows; red to dark blue, downflows) in the subsurface layers of the simulation. The center panel shows the radial magnetic field (gray to yellow, outward-directed magnetic field; gray to green, inward-directed) at the same depth. Note how the characteristic spa-

tial scales are the same for both quantities, which also evolve on the same temporal scale of days. The right panel shows the magnitude of the zonal magnetic field component, deep in the interior of the simulation, at the base of the convection layer. Note the banded structure at mid-latitudes, roughly symmetric about the rotation axis. This torus-like structure, and its opposite-polarity counterpart in the other hemisphere, undergo synchronous polarity reversals on a cadence of about 40 years.

Maxwell's laws of electromagnetism. The challenge lies with the strong nonlinearities characterizing the interactions of fluid flows and magnetic fields, as well as the extremely wide range of spatial and temporal scales over which these interactions take place under solar interior conditions.

Although the induction of intense magnetic fields by turbulent convection occurs readily in these numerical simulations [(6); (7) and references therein], it has proven much harder to establish the conditions allowing the self-organization of the spatiotemporally intermittent turbulent magnetic field into structures that maintain their spatial and temporal coherence over scales much greater than those of turbulent convection. The figure shows a snapshot of a simulation in which this self-organization has taken place. In the outer layers (left and center panels), the flow and magnetic field have roughly the same spatial scale; however, deep within the simulation domain (right panel), at the base of the turbulent convection layer, zonal bands of strong magnetic field are built up, parallel to and antisymmetric about the equatorial plane. This is precisely the type of internal magnetic field configuration believed to be conducive to the production of sunspots [(8) and references therein], the largest and most strongly magnetized structures found at the solar surface.

In the specific simulations displayed, the zonal magnetic field bands reverse their polarity quite regularly, every 40 years or so. This is longer than the observed solar cycle by a factor of  $\sim 4$ , but the very fact that a reg-

ular cycle is produced is already remarkable. Other numerical simulations, in principle quite similar in their overall design and parameter regimes, produce instead large-scale zonal structures within the turbulent convection layer, peaking at low latitudes, sometimes reversing their polarity on time scales of a few years, in other cases remaining steady. In these various simulations the internal flows are far more alike than the very different magnetic cycles emerging from their inductive action. Where the various simulation models differ is at the level of what one would normally hope to be details: grid resolution, the numerical treatment of dissipation at the smallest resolved scale, the presence of convectively stable fluid underlying the convecting layers. Evidently, something essential is hiding in these details.

If the Sun's large-scale magnetic field manages to remain quasi-steady on time scales much longer than that of convection, then a near-balance must exist between the inductive contributions of turbulent and large-scale flows (9, 10), because dissipation is inefficient at these larger scales. The small residual induction is then what is driving the much slower cyclic evolution of the large-scale magnetic component (11). This delicate balance is most certainly influenced by the manner in which dissipation is implemented at the smallest spatial scales resolved in the simulations, and therein may lie some of the aforementioned sensitivity to "details." Other physical mechanisms that have received little attention may also be in play. Simulations similar to that shown in the figure (also see

supplementary materials) reveal that accumulation of magnetic fields beneath the base of the convection zone may become prone to MHD instabilities triggered at those depths, which may in turn destabilize the cycle on very long time scales. Such instabilities may even play a role in timing the regular polarity reversals. Investigation of these mechanisms is a painstaking task, in view of the enormous volume of simulation data that must be stored, visualized, analyzed, and ultimately understood in physical terms. The real work is just beginning.

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#### Supplementary Materials

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# Optogenetic Dissection of Entorhinal-Hippocampal Functional Connectivity

Sheng-jia Zhang,\*† Jing Ye,\* Chenglin Miao, Albert Tsao, Ignas Cerniauskas, Debora Ledergerber, May-Britt Moser, Edvard I. Moser†

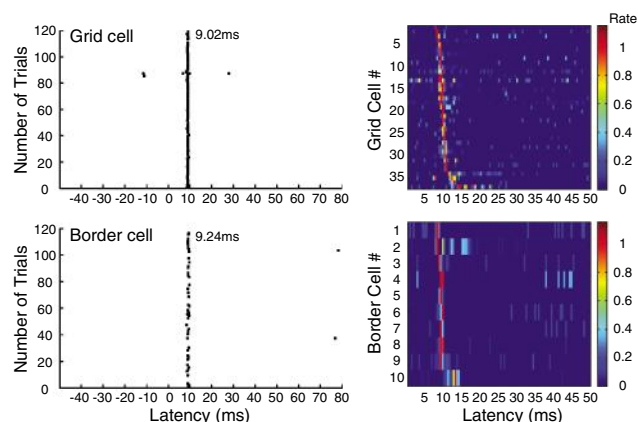
**Introduction:** The mammalian space circuit contains several functionally specialized cell types, such as place cells in the hippocampus and grid cells, head-direction cells, and border cells in the medial entorhinal cortex (MEC). The interaction between entorhinal cell types and hippocampal place cells is poorly understood. Hippocampal place fields are thought to be generated by transformation of spatial signals from the MEC, but which cell types contribute to this process remains elusive.

**Methods:** We used a combined optogenetic-electrophysiological strategy to determine functional identity of entorhinal cells with output to place cells in the rat hippocampus. Channelrhodopsin-2 (ChR2) was expressed selectively in the hippocampus-targeting subset of entorhinal projection neurons by injecting retrogradely transportable ChR2-coding recombinant adeno-associated virus (rAAV) into the hippocampus. Hippocampus-projecting MEC cells could then be identified, after expression of ChR2 transgenes, as neurons that responded reliably, at a minimal latency, to photostimulation of either cell bodies in the MEC or axons of MEC cells in or near the hippocampus.

**Results:** Many light-responsive MEC cells were grid cells, but short-latency firing could also be induced in border cells and head-direction cells, as well as neurons with irregular firing fields or no fields at all. In each cell group, the majority of neurons discharged at minimal response latencies, suggesting

that they had direct projections to the hippocampus. The same cells could also be backfired, at slightly longer latencies, by photostimulation of the cells' axons in the hippocampus. MEC cells could also be activated by photostimulation in the hippocampal CA1 subfield, but response latencies were more than 150% longer than with somatic or axonal stimulation, suggesting the activation was now synaptic.

**Discussion:** These findings suggest that place signals are generated by convergence of signals from a variety of entorhinal functional cell types, of which grid cells are the most abundant spatial cell type. A dual spatial



**Grid cells and border cells fire at similar latencies in response to local photostimulation.** (Left) Spike rasters showing discharge in response to successive 3.5-ms light pulses for a grid cell and a border cell. Dots indicate spike times. (Right) Spike rasters showing color-coded normalized firing rate 0 to 50 ms after the light pulse for all grid cells and border cells.

input from grid cells and border cells is consistent with the idea that place cells have access to both self-motion and landmark-based information and raises the possibility that the spatial metric of the place-cell population originates from grid cells, whereas boundary- and landmark-induced firing is derived directly from border cells. The dual nature of the spatial input may explain the observation that place cells precede mature grid cells during ontogenesis of the spatial representation system and that place cells can maintain location specificity under conditions that reduce grid-cell periodicity in adult rats. Convergent input from a broad spectrum of entorhinal cell types may also enable individual place cells to respond dynamically, so that different types of input are favored in different behavioral states or circumstances.

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## FIGURES IN THE FULL-TEXT VERSION

- Fig. 1. rAAV-induced retrograde labeling of hippocampus-projecting MEC neurons
- Fig. 2. Sagittal brain sections showing distribution of ChR2-expressing neurons in the MEC of a rat injected with rAAV-ChR2-FLAG
- Fig. 3. Photoexcitable cells in dorsal MEC
- Fig. 4. Firing latencies for the entire sample of photoresponsive MEC neurons
- Fig. 5. Effect of power density on response latency for a representative light-responsive MEC cell
- Fig. 6. Photoinduced spiking in putative GABAergic neurons
- Fig. 7. Synaptic discharge of MEC neurons after stimulation of hippocampal pyramidal cells
- Fig. 8. Light-induced discharge at higher stimulation frequencies
- Fig. 9. Antidromic discharge of MEC neurons after photostimulation of entorhinal projections to the hippocampus

## SUPPLEMENTARY MATERIALS

<http://www.sciencemag.org/content/340/6128/1232627/suppl/DC1>

Figs. S1 to S21

Full References

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### Perspective

B. Poucet, F. Sargolini, A trace of your place. *Science* **340**, 35 (2013). DOI: 10.1126/science.1237567

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# Optogenetic Dissection of Entorhinal-Hippocampal Functional Connectivity

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We used a combined optogenetic-electrophysiological strategy to determine the functional identity of entorhinal cells with output to the place-cell population in the hippocampus. Channelrhodopsin-2 (ChR2) was expressed selectively in the hippocampus-targeting subset of entorhinal projection neurons by infusing retrogradely transportable ChR2-coding recombinant adeno-associated virus in the hippocampus. Virally transduced ChR2-expressing cells were identified in medial entorhinal cortex as cells that fired at fixed minimal latencies in response to local flashes of light. A large number of responsive cells were grid cells, but short-latency firing was also induced in border cells and head-direction cells, as well as cells with irregular or nonspatial firing correlates, which suggests that place fields may be generated by convergence of signals from a broad spectrum of entorhinal functional cell types.

Neuronal systems investigations have matured to the point where computation can be understood at the microcircuit level. In sensory systems, electric signals can be followed through networks of increasing complexity, from receptors to cortex, and experiments have established how neural representations change from one level to the next. Less is known, however, about how neural codes are formed and transformed in higher-order nonsensory cortices. One example of a high-level neural representation is the place code of the hippocampus (1, 2). Place cells are hippocampal cells that fire when the animal is at a particular location in a particular environment, independently of specific sensory inputs (3). Almost all pyramidal cells in the CA areas of the hippocampus are place cells (4–6). The origin of the place signal has not been established, but the recent identification of functionally specialized cell types in the medial entorhinal cortex (MEC), upstream of the place cells, provides some clues (2). The first entorhinal cell type to be characterized was the grid cell, which has periodic firing fields that span the entire available space in a hexagonal pattern (7, 8). Grid cells intermingle with head-direction cells, which fire whenever animals face a particular direction, irrespective of where they are or what they are doing (9–11), as well as “border cells,” which signal proximity to specific geometric boundaries of the local environment (11–13). The entorhinal cortex also contains cells with irregular spatial firing fields or no spatial firing fields

at all (10–12, 14). All of these cell types are active in all environments, and their spatial and directional firing relationships are preserved when the animals move from one environment to another (12, 15), which suggests that these cells, unlike the place cells of the hippocampus (16), are part of a path integration-dependent metric applied universally across environments (2, 8, 17).

Although the entorhinal and hippocampal cortices are known to be strongly connected, the functional interactions between the two mapping systems, as well as the interdependence between the different cell types, have not been determined. Hippocampal place fields may be generated by transformation of spatial signals from the entorhinal cortex (2, 17–23), but which cell types contribute to this process remains elusive, given that only a subset of the entorhinal projection neurons target the hippocampus (24). The present study set out to identify the functional cell types that project to the hippocampus by combining tetrode recording in the MEC with optogenetic tagging of the hippocampus-projecting cell population in this region.

## Results

### Retrograde Transduction of Entorhinal Projection Neurons

Entorhinal neurons with projections to the hippocampus can be identified by antidromically stimulating axons in the projection area at the same time as spikes are recorded from the parent cells in the entorhinal cortex. The power of this strategy is limited, however, by the small fraction of entorhinal-hippocampal fibers that can be discharged from a single electrode position in the hippocampal terminal region (25). A substantially larger proportion might be reached if it

were possible to tag the incoming axons by hippocampal infusion of a widely diffusible viral vector carrying a light-responsive transgene. We used recombinant adeno-associated virus (rAAV) to target transgenes to entorhinal axon terminals across large parts of the dorsal hippocampus. To maximize transgene expression in the cell bodies of the projection neurons, we improved the retrograde axonal transport properties of classic rAAV-based transduction protocols (26, 27).

Transduction potency, transgene expression, and retrograde transport efficiency were evaluated by stereotaxic injections of rAAV2/1-EYFP-Nuc into the rat dorsal hippocampus (EYFP, enhanced yellow-green fluorescent protein) (28). Two to 3 weeks after viral injection, robust labeling of EYFP fluorescence could be observed in all subfields of the dorsal hippocampus at and around the injection sites (Fig. 1). In addition, EYFP-positive neurons could be identified in layers II and III of MEC, which indicates that the rAAV2/1 vector had infected cell bodies of MEC neurons with projections to the hippocampal injection site. EYFP-positive neurons were nearly absent in the deep layers of MEC (Fig. 1 and fig. S1), which indicates that entorhinal transduction was not caused by passive diffusion of injected virus from the hippocampal infusion site. Labeling was also observed in other brain regions with projections to the hippocampus (29), such as the parasubiculum, lateral entorhinal cortex, nucleus reuniens, and claustrum (Fig. 1). The EYFP expression pattern in superficial layers of the MEC was replicated in an animal injected with rAAV coding for the reporter gene  $\beta$ -galactosidase ( $\beta$ -gal or LacZ) fused with a C-terminal FLAG tag (fig. S2).

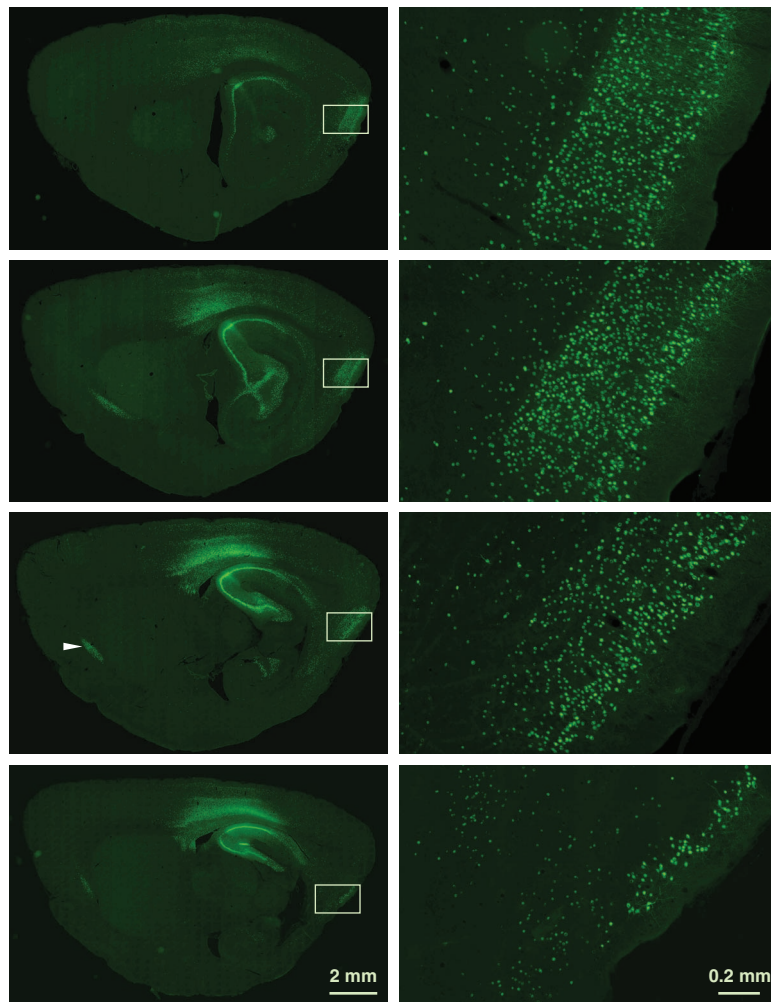
### Targeting Channelrhodopsin-2 (ChR2) to Entorhinal Projection Neurons

To obtain optogenetic control of the virally transduced MEC neurons, we engineered rAAV2/1 encoding the gain-of-function (H134R) photocurrent-enhanced mutant (30, 31) of the light-activated cation channel ChR2 (32–34). EYFP was replaced with a nonfluorescent FLAG tag in this vector. The rAAV was stereotactically injected into the dorsal hippocampus. Immunohistochemical staining with FLAG antibody showed that, within 2 to 3 weeks after intrahippocampal injection, there was reliable widespread expression of the transgene in all subfields of the dorsal hippocampus, particularly the CA fields, as well as in superficial layers of the MEC (28 animals with rAAV-ChR2-FLAG) (Fig. 2 and fig. S3). FLAG was expressed widely across the somatodendritic surface membrane of the MEC cells (Fig. 2 and fig. S3, right panels). The tetrodes were always located near FLAG-expressing neurons (Fig. 3 and fig. S3). A similar retrograde-transport strategy was developed to transduce entorhinal projection neurons with genes for microbial opsins that hyperpolarize the cell in response to local photostimulation (NpHR and Arch) (figs. S4 to S7) (35).

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**Fig. 1. rAAV-induced retrograde labeling of hippocampus-projecting MEC neurons.** Sagittal brain sections show viral transduction of hippocampus-projecting cells in the MEC of a rat injected with EYFP-coding rAAV in the dorsal hippocampus (all sections from the same rat; top, lateral; bottom, medial). **(Left)** Low magnification; **(right)** high magnification of framed area at left. Note high density of EYFP-expressing neurons in layers II and III but not V and VI in the dorsal MEC **(right)**. Arrowhead indicates retrogradely labeled cells in the claustrum, which has massive unidirectional projections to the hippocampus (63).

Injections in the overlying neocortex did not result in labeling in MEC (fig. S8).

#### Photoexcitation of Entorhinal Projection Neurons

To identify the subset of entorhinal neurons with direct projections to the hippocampus, we used a photoexcitation strategy where direct and indirect activation could be distinguished based on response latencies. ChR2-coding rAAV2/1 was first injected into the hippocampus and then, during the same surgical session, an assembly of tetrodes and a laser-coupled optical fiber was implanted into the MEC (fig. S3). Several weeks later, spike activity was recorded from cells in layers II and III of MEC [665 putative principal cells and 114 high-rate narrow-spiking cells likely to be  $\gamma$ -aminobutyric acid-releasing (GABAergic) interneurons] while the animals ran to collect food morsels in a 1-m-wide square enclosure. Grid cells, border cells, and head-direction cells were defined, respec-

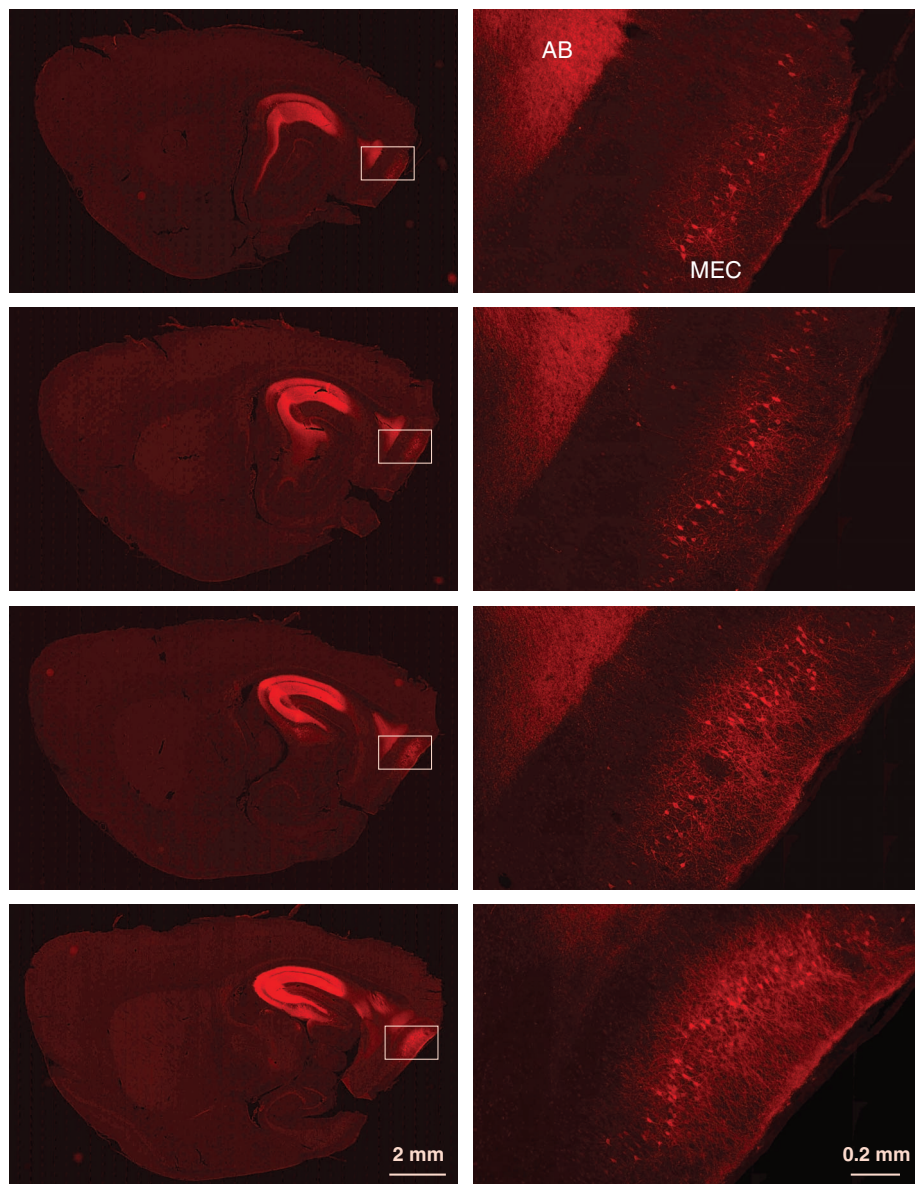
tively, as cells with scores for six-fold rotational symmetry, border proximity, and directional modulation that exceeded the 99th percentile for a distribution of corresponding scores from shuffled data (4, 5, 11) (figs. S9A, S10A, and S11A). Spatially modulated cells with irregular nonperiodic firing fields (10–12, 14) were defined as cells that did not satisfy criteria for grid cells, border cells, or head-direction cells but had spatial information values that exceeded the 99th percentile of the shuffled data (fig. S12A). Nonspatial cells were defined as cells that satisfied neither of the above criteria. Using these definitions, we found that 158 cells were grid cells (71 clearly in layer II, 60 clearly in layer III); 41 were border cells (12 clearly in layer II, 18 clearly in layer III); 166 were head-direction cells (5 clearly in layer I, 44 clearly in layer II, 75 clearly in layer III); 47 were irregular spatial cells (26 clearly in layer II, 16 clearly in layer III); and 253 were nonspatial (118 clearly in layer II, 100 clearly in layer III).

Many head-direction cells had conjunctive properties, satisfying criteria for grid cells or border cells in addition (9.0% and 11.4%, respectively, with a 99th percentile threshold).

For each recorded MEC neuron, we then asked if the cell discharged in response to local photostimulation at a 473-nm wavelength, as might be expected if it expressed ChR2. Photostimulation was given after the foraging trial while the rat rested in a deep flower pot on a pedestal. After a 2-min no-stimulation baseline, the rat received 2 min of shuttered 3.5-ms 473-nm pulses at a light power density of  $10 \text{ mW/mm}^2$  and a frequency of 1 Hz, followed, in 16 of the rats, by 2-min blocks of 5-pulse trains at 5 Hz and 10-pulse trains at 20 Hz (train rates 0.2 Hz). Photostimulation did not induce detectable changes in the spike waveforms of the recorded cells under the described conditions (fig. S13). A large fraction of the cells responded to the photostimulation (Figs. 3 and 4). Light-responsive cells were identified and counted by comparing, for each cell, the proportion of spikes emitted after the light stimulus at 1 Hz stimulation with the proportion expected to fire at similar latencies by chance. The expected proportion was determined for each cell by a shuffling procedure in which the timing of each spike was displaced randomly forward or backward in time within an interval ( $-100 \text{ ms}$  to  $100 \text{ ms}$ ) around the light stimulus. The displacement was repeated 10,000 times. Spikes were then counted for bins of 1 ms in 100-ms time windows after the stimulation, both for the real data and for the aggregated shuffled data, and the 3-ms block with the maximum number of spikes was identified for each of the two distributions. The cell was defined as light responsive if the proportion of spikes in the most-active 3-ms block in the real data exceeded the 99.9th-percentile value for the proportion of spikes in the most-active 3-ms block in the shuffled distribution. On the basis of this criterion, a total of 183 MEC cells were defined as photoresponsive (47.5% of all cells recorded in MEC layers II and 23.5% of all cells in layer III). A total of 135 of these neurons had waveform and firing-rate properties thought to be typical of principal cells (figs. S13 and S14A). The number of spikes emitted at the peak latency in the responsive cells was many times higher than the background firing at any other time (Figs. 3 and 4 and figs. S9 to S12 and S14).

The response latency of the cells that passed the criterion was short and varied minimally within and between animals (Fig. 4 and fig. S15). The modal latency of all responsive principal cells at the standard power density ( $10 \text{ mW/mm}^2$ ) was  $9.50 \pm 0.14 \text{ ms}$ , measured from the beginning of the light pulse (mean  $\pm$  SEM across cells). This latency is comparable to the 9- to 10-ms response time obtained with similar light flashes in ChR2-activated principal neurons of other cortical regions (36). Response latencies varied minimally with changes in power density above  $\sim 2.5 \text{ mW/mm}^2$  (Fig. 5 and fig. S16) (interquartile ranges of 9.9 to 10.6 ms at  $2.5 \text{ mW/mm}^2$ , 9.5 to





**Fig. 2. Sagittal brain sections showing distribution of ChR2-expressing neurons in the MEC of a rat injected with rAAV-ChR2-FLAG.** (Left) Low resolution; (right), high resolution of the framed area. ChR2-expressing neurons were visualized by immunofluorescent staining using a fluorescent conjugated secondary antibody against the primary antibody to FLAG. Note widespread expression in superficial layers of the dorsal MEC. Strong labeling is also seen in the angular bundle (AB), which contains the perforant-path and temporoammonic fibers of hippocampus-targeting entorhinal projection cells (Fig. 9 and fig. S21). In the hippocampus, all strata are heavily labeled, reflecting expression in both cell bodies and axons.

10.5 ms at 5.0 mW/mm<sup>2</sup>, and 9.1 to 10.2 ms at 10 mW/mm<sup>2</sup>). The relative constancy of response latencies across trials and cells with the chosen stimulation parameters points to photoillumination-induced discharge as a powerful tool for identifying cells with monosynaptic connections to a ChR2-infected target region.

A large fraction of the photoresponsive principal cells were grid cells (37 cells or 27.4%), but the sample of excitable principal cells also included 10 border cells (7.4%) and 16 head-direction cells (11.9%), as well as three irregular spatial cells (2.2%) and 69 nonspatial cells

(51.1%) (Figs. 3 and 4 and figs. S9 to S12). Among the excitable head-direction cells, three had conjunctive properties (one grid × head direction; three border × head direction). Responsive grid cells were recorded in both layer II and layer III (grid cells: 22 and 9 cells, respectively; border cells: 5 and 1 cell; head-direction cells: 1 and 5 cells; irregular spatial cells: 1 in each layer; nonspatial cells: 42 and 14 cells, respectively). Because the counts of nonresponsive cells are likely to include hippocampus-projecting cells outside the illuminated region, we followed up with analyses where the sample was limited to

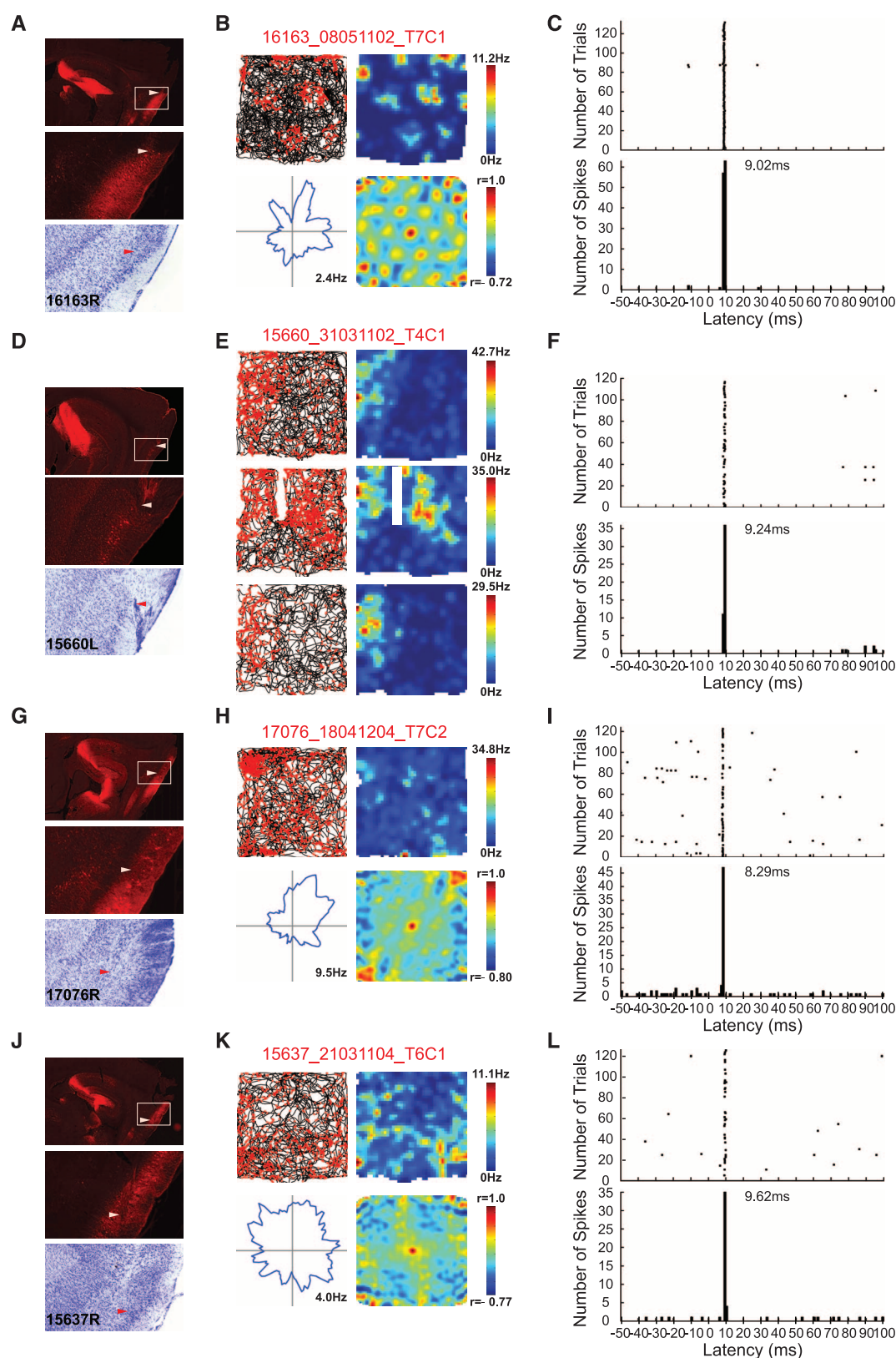
responsive and nonresponsive cells from tetrode bundles with at least two responsive cells. Within this subset, likely to be nearer the light source, the five cell types responded at approximately similar frequencies (grid cells: 24 of 66 cells, or 36.4%; border cells: 7 of 13 cells, or 53.8%; head-direction cells: 6 of 36 cells, or 16.7%; irregular spatial cells: 0 of 23 cells; nonspatial cells: 41 of 80 cells, or 51.3%). The exact proportion of inputs from each cell class cannot be inferred, considering that uptake and retrograde transport of virus may be influenced by possible differences in terminal distribution, axonal diameter, and myelination.

The functional identity of putative border cells was verified on separate trials where a 50-cm-long barrier was inserted centrally in the recording box in parallel with the wall that maintained the original border field (Fig. 3E and fig. S10B). In cells with high border scores, this generally leads to the emergence of a new field along the insert on the side that faces away from the original field (the distal side) (12, 37, 38). In the present border cells, the firing rate increased from  $0.64 \pm 0.11$  to  $7.1 \pm 1.3$  Hz within a 20-cm band on the distal side of the wall insert [ $t(6) = 5.18$ ,  $P < 0.005$ ; paired-samples  $t$  test], whereas no increase was detected on the proximal side [from  $3.6 \pm 1.3$  to  $3.1 \pm 0.8$  Hz;  $t(6) = 0.83$ ,  $P > 0.40$ ]. Firing rates returned to baseline after removal of the wall ( $1.2 \pm 0.5$  Hz on the distal side).

#### Direct Activation of ChR2-Expressing Cells

A photoresponsive cell might fire because ChR2 channels are activated, not in the recorded cell itself, but in cells with synaptic connections to the recorded cell. Such connected cells might include local entorhinal cells as well as hippocampal cells with axons branching into the photostimulated region of the MEC (39). We first evaluated the presence of indirectly activated neurons by measuring the variation in latency from stimulation to discharge in the entire population of responsive cells. The minimum modal latency in the cell sample should correspond to the time it takes to elicit an action potential by direct illumination of the recorded cell, whereas longer latencies could reflect indirect activation. In the majority of MEC cells, local photostimulation caused firing only at short latencies, with little variation from cell to cell or from trial to trial (Fig. 4 and figs. S9 to S12). Short latencies predominated in all functional cell types. Delayed firing and secondary firing peaks were observed only in exceptional cases (figs. S9C, S10C, S14, and S17). The number of long-latency outliers varied slightly between the groups, but the values for the lower 10th, 20th, and 50th percentiles were highly similar (Fig. 3) (grid cells: 8.69, 9.2, and 9.69 ms, respectively; border cells: 8.63, 8.86, and 9.31 ms; head-direction cells: 8.30, 8.35, and 9.18 ms; irregular and nonspatial cells: 7.87, 8.46, and 9.41 ms), which suggests that cells with direct hippocampal projections exist in all functional cell populations. The latency distributions of most cells were symmetric, with skewness values not

**Fig. 3. Photoexcitable cells in dorsal MEC.** (A to C) grid cell; (D to F) border cell; (G to I) head-direction cell; (J to L) nonspatial cell. (A) Chr2-expressing neurons in MEC after rAAV-ChR2-FLAG transduction in the dorsal hippocampus. Chr2-expressing neurons were visualized by immunofluorescent staining using a fluorescent conjugated secondary antibody against the primary antibody to FLAG. (Top) Sagittal brain section showing FLAG expression in hippocampus (left) and dorsal MEC (framed area to the right); (middle) magnification of the framed area; (bottom), adjacent Nissl-stained section corresponding to the high-magnification FLAG stain. End of tetrode trace is indicated (arrow-head, approximate recording position). Rat number and hemisphere (L, left; R, right) are indicated. (B) Firing pattern of a responsive grid cell recorded at the tetrode location in (A). Text label indicates rat number, recording trial, and cell number (T, tetrode; C, cell). (Top left to bottom right): Trajectory with superimposed spike locations in red; color-coded rate map with scale bar indicating minimum and maximum firing rates; polar plot showing firing rate as a function of head direction, with peak rate indicated; and color-coded auto-correlation matrix for the rate map with scale bar showing minimum and maximum correlation values. The scale of the autocorrelogram is twice the scale of the rate map. (C) (Top) Spike rasters showing discharge in response to successive light stimuli for the grid cell in (B) from 50 ms before the stimulus to 100 ms after. The optic fiber was dorsal to the tetrode in MEC. Light was on from 0 to 3.5 ms. Each row corresponds to the beginning of a 1-s period. Dots indicate spike times. Note reliable discharge at a fixed latency (9.0 ms) after the onset of stimulation. (Bottom) Peristimulus spike histograms showing distribution of spikes during the poststimulus interval. (D) Location of tetrodes and virally transduced neurons in MEC in an animal with a light-responsive border cell [symbols as in (A)]. (E) Firing pattern of a photoresponsive border cell recorded at the tetrode location indicated in (D). (Left) Trajectories with spike locations; (right) color-coded rate maps. The first trial (top) was recorded in an empty 1-m-wide square box. In the subsequent trial (below), a wall (white stripe) was placed in the box, in parallel with the peripheral wall that maintained the border field when the box was empty. On the third trial, the rat was tested again in the empty box. The creation of a new field on the distal

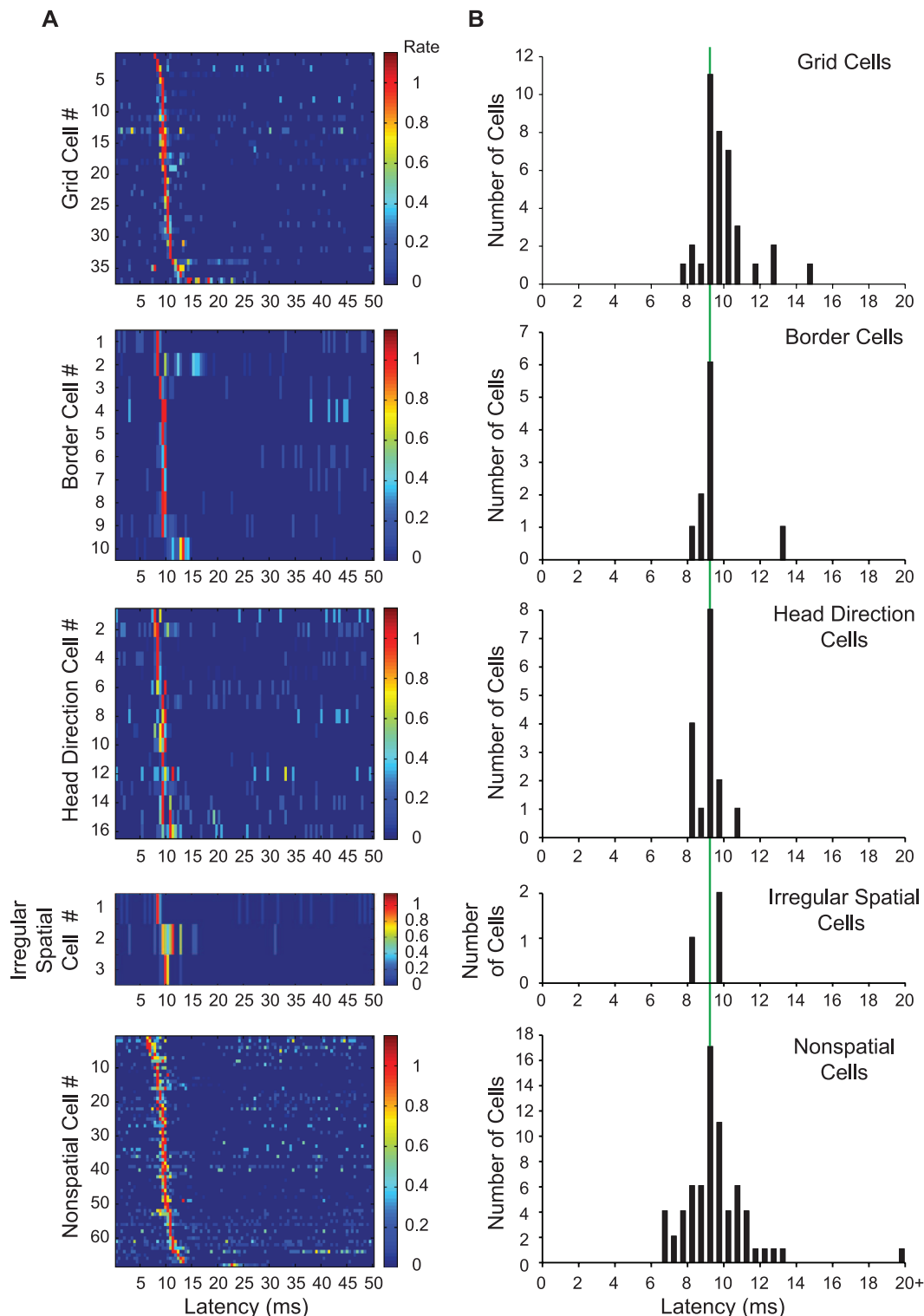


side of the insert, with an orientation matching the original field along the peripheral wall, is a defining characteristic of border cells in the MEC (12, 37). (F) Spike rasters and peristimulus spike histogram as in (C). (G to I) and (J to L) Location of tetrodes and virally transduced neurons, spatial firing patterns, and rasters and peristimulus spike histograms, as in (A to C).



**Fig. 4. Firing latencies for the entire sample of photoresponsive MEC neurons.**

**(A)** Spike rasters showing color-coded firing rate as a function of time after the start of photostimulation for all categories of principal cells that responded to the light pulse. Firing rate is shown for successive time bins of 0.5 ms during the 50 ms after the light pulse. Each row shows data for one cell. Dark blue is silent; red is high rate; and rates are normalized to peak rate (color scale at right). Among the head-direction cells, number 11 is a conjunctive grid  $\times$  head-direction cell, and numbers 3, 8, and 13 are conjunctive border  $\times$  head-direction cells. The absence of spike latencies shorter than  $\sim 9$  ms suggests that this is the time it takes to discharge a Chr2-expressing principal cell by direct optical stimulation under the conditions described herein (36). The almost identical minimal discharge latency of the four cell groups suggests that all groups have direct projections to the hippocampus. The few cells with longer latencies (e.g., grid cells numbered 35 to 37) were probably activated indirectly (synaptically). **(B)** Distribution of modal peak latencies across bins of 0.5 ms during the first 20 ms after the onset of photostimulation (all cells of each category). Green vertical line indicates modal latency for all principal cells.



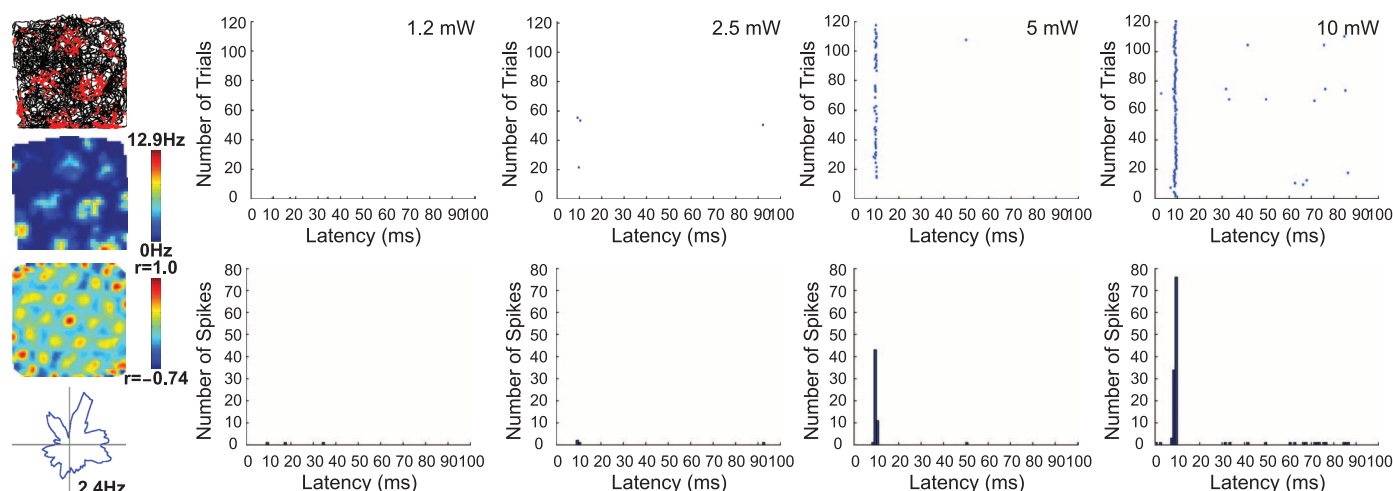
differing from zero in any of the groups (grid cells:  $-0.28 \pm 0.25$ ; border cells:  $-0.58 \pm 0.40$ ; head-direction cells:  $-0.47 \pm 0.47$ ;  $t$  values  $< 1.5$ ,  $P > 0.18$ ), except for the nonspatial cells [ $-0.49 \pm 0.18$ ;  $t(67) = 2.7$ ,  $P < 0.01$ ]. The general lack of positive skew in the latency distributions of individual cells supports the idea that the vast majority of light-induced responses were directly activated. The low number of long-latency cells

is consistent with the lack of direct excitatory connections in layer II of MEC (40, 41).

The consistently short activation latencies of the space- and direction-modulated principal cells were in contrast to the variable responses obtained in light-responsive MEC cells with discharge characteristics similar to those of hippocampal interneurons. These cells had short waveforms (peak-to-trough width:  $177 \pm 7 \mu\text{s}$ ),

high firing rates (mean rate:  $19.7 \pm 1.1$  Hz), and no clear spatial or directional preferences (Fig. 6, A and B, and fig. S14), properties which all are thought to be typical for fast-spiking GABAergic cells in the hippocampus as well as the entorhinal cortex (42, 43). Forty-eight of the 114 high-rate narrow-waveform units in the cell sample (42.1%) satisfied the criterion of having a larger number of spikes in the most-active 3-ms block after

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**Fig. 5. Effect of power density on response latency for a representative light-responsive MEC cell.** The example cell is a grid cell (5-digit rat number, trial number, and cell number are indicated). (Left column, from top to bottom) Trajectory with spike locations, color-coded rate map, directional rate map, and autocorrelation map, as in Fig. 3B. (Right four columns): (Top row) Raster diagram (as in Fig. 3C) showing firing in re-

sponse to light pulses at increasing power densities, from 1 mW/mm<sup>2</sup> to 10 mW/mm<sup>2</sup>. Light was on from 0 to 3.5 ms in each case. (Bottom row) Peristimulus time histogram. Note reliable discharge at an almost fixed latency (~9 to 10 ms) at 5 and 10 mW/mm<sup>2</sup>. Few cells discharged at less than 5 mW/mm<sup>2</sup>, but when they discharged, the latencies were most often similar to those of the higher power densities.

illumination than in the corresponding shuffled distribution. Almost half of these cells discharged at latencies similar to those of the principal cell groups (less than ~10 to 11 ms) (Fig. 6, C to E), which is consistent with previous studies showing that a subset of entorhinal GABAergic neurons has direct projections to the hippocampus (44, 45). However, as a whole, the latency distribution was skewed toward longer latencies, with more than one-quarter of the population firing between 11 and 16 ms and the rest within a broad band ~20 to 40 ms after the light stimulus (Fig. 6, D to E). The mean firing latency of the putative GABAergic projection neurons ( $12.8 \pm 1.0$  ms) was significantly longer than for the space and direction-modulated principal cells [ $9.6 \pm 0.1$  ms;  $t(112) = 3.7$ ,  $P < 0.001$ ]. Medians were also different (10.4 versus 9.4 ms; Mann-Whitney  $U$  test:  $Z = 3.9$ ,  $P < 0.001$ ). The fact that so many late-activated cells were found in this cell group is consistent with the observation that the majority of local connections from projection neurons in MEC layers II and III are onto inhibitory cells (41).

The symmetric distribution of response latencies in the principal neurons also speaks against the possibility that cells were activated by stimulation of ChR2-expressing axons from projection neurons in the CA1 of the hippocampus. Output from CA1 cells would be expected to skew the response latencies of the MEC cells to the right. To test the contribution of hippocampal axons more directly, we implanted optic fibers in the CA1 pyramidal cell layer or the stratum oriens or alveus of five animals with tetrodes in the MEC. We identified 14 MEC cells that responded to CA1 illumination in these animals (seven principal cells and seven high-rate narrow-waveform cells) (Fig. 7 and fig. S18). These neurons had

peak latencies ranging from 15.8 to 30.2 ms. In five of the cells, stimulation was evoked both from the hippocampus and the MEC, always with longer latencies after hippocampal illumination (9.7 to 10.7 ms versus 18.5 to 29.4 ms).

In a further test of whether photostimulated MEC cells were directly activated, we determined if the responses of such cells are maintained at high-pulse frequencies where synaptically triggered discharges become unreliable (46). The predominance of 9- to 10-ms latencies was maintained at 5 and 20 Hz in all spatial and directional cells in 16 animals (Fig. 8A and fig. S19). The percentage of pulses that evoked spikes at these frequencies, from the second pulse and onward, was not lower at 5 and 20 Hz than at 1 Hz in any of the cell types (Fig. 8B). The modal spike latencies of grid cells, border cells, and head-direction cells remained unchanged across stimulation frequencies (Frequency:  $F_{2,60} = 0.32$ ; Frequency  $\times$  Cell Type:  $F_{4,60} = 0.14$ ; repeated-measures analysis of variance).

We also tested, in a small sample, whether firing could still be induced optically after blockade of excitatory neurotransmission in the recording area. Three rats were implanted with cannulae above the fiber-tetrode bundle in the MEC. Once light-responsive neurons were identified in MEC, a cocktail of the competitive AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and the competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist D(-)-2-amino-5-phosphonopivalic acid (D-APV) was infused to interrupt glutamatergic neurotransmission at the recording location. In six cells, we were able to follow neural activity until firing recovered after washout of the drug (fig. S20). The infusion substantially reduced all sponta-

neous firing, but short-latency firing could still be evoked. Evoked activity decreased in some of the cells, as expected if glutamatergic blockade changes excitability by removing excitatory drive within the network, but the relative persistence of short-latency discharges is consistent with the interpretation that the overwhelming majority of light-responsive entorhinal principal cells were activated directly.

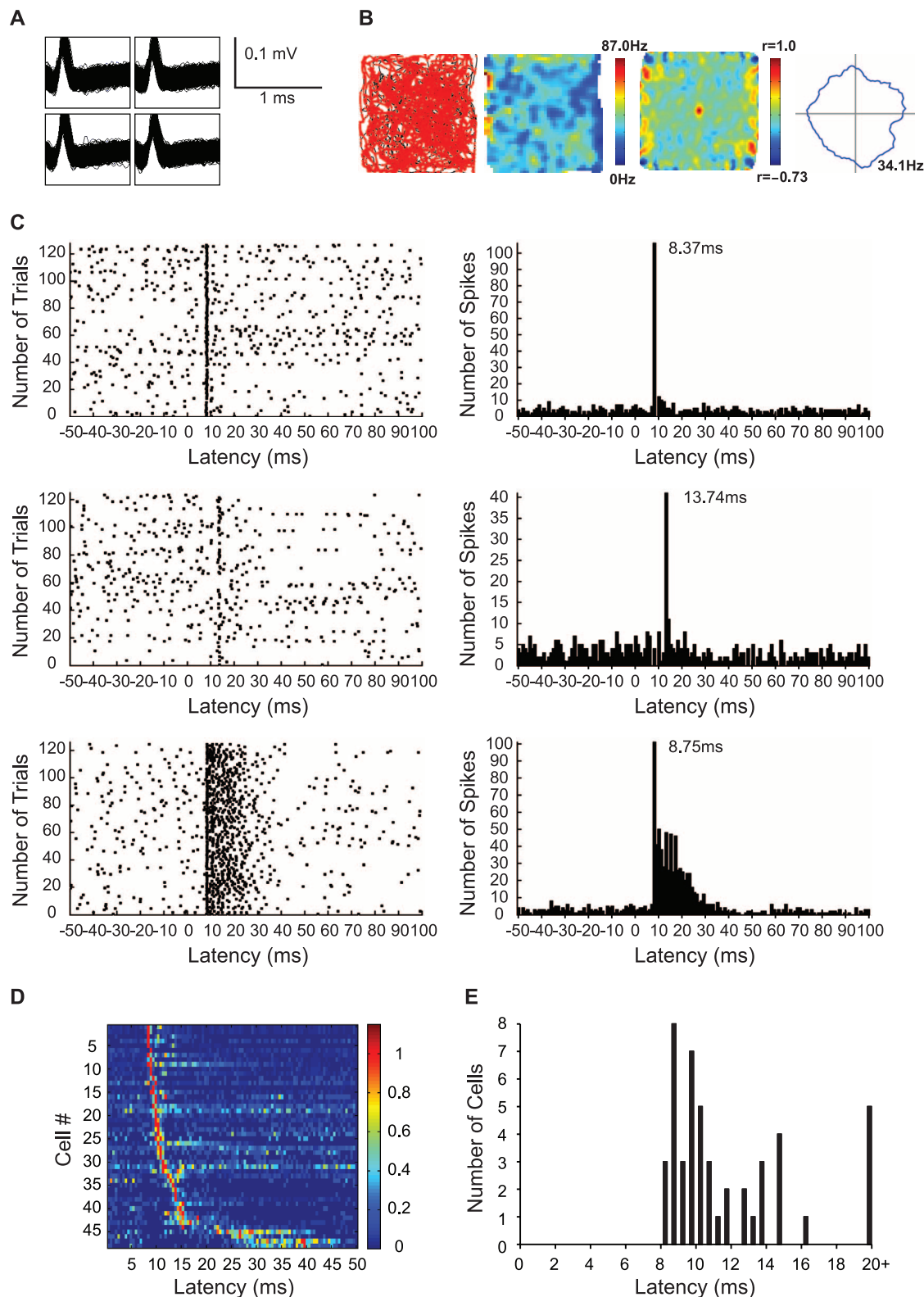
Finally, to confirm the functional diversity of the entorhinal direct inputs to the hippocampus, we adapted an optogenetic backfiring approach (47) for use in *in vivo* studies. MEC neurons with axons in the hippocampus were photostimulated either in the perforant path–projection zone within the hippocampus or in the angular bundle posterior to the hippocampus, upstream of the point where axons of MEC cells fan into the hippocampus (Fig. 2). We were able to backfire five grid cells, two border cells, four nonspatial cells, and three high-rate narrow-waveform cells in four animals (Fig. 9 and fig. S21). Latencies were 1 to 2 ms longer than with local entorhinal stimulation, as expected because of the added conduction latencies. With stimulation in the perforant-path zone, the mean firing latency ( $\pm$  SEM) was  $12.7 \pm 0.1$  ms; with stimulation in the angular bundle the latency was  $11.6 \pm 0.1$  ms. The latencies were significantly longer with stimulation in the hippocampus than in the angular bundle [ $t(12) = 6.5$ ,  $P < 0.001$ ].

## Discussion

We have shown that optogenetics can be combined with electrophysiological recording to identify the projection pattern of functionally defined cell types in intermingled neural populations of the mammalian spatial representation circuit. Building



**Fig. 6. Photoinduced spiking in putative GABAergic neurons.** (A) Superimposed waveforms from a high-rate narrow-waveform MEC neuron. Representative waveforms are shown for each of the four electrodes of the tetrode. (B) (Left to right) Trajectory with spike locations, color-coded rate map, directional rate map, and autocorrelation map for a representative high-rate narrow-waveform cell, as in Fig. 3B. (C) Photostimulation-induced discharge in three representative cells in this group, as in Fig. 3C (spike rasters and peristimulus spike histograms). Modal peak latencies are indicated for each cell. The cell in the top panel of (C) is the same as the one in (B). The response latency of this cell is similar to that of the principal neurons in Fig. 4. The middle cell fired at a longer latency, which suggests that it was activated synaptically. The bottom cell fired at a short latency but showed additional peaks during the subsequent 10 ms, which suggests that the cell was discharged both directly and indirectly. (D) Color-coded spike rasters for the entire sample of responsive high-rate narrow-waveform units, as in Fig. 4A. (E) Distribution of modal peak latencies across bins of 0.5 ms during the first 20 ms after the onset of local photostimulation (all high-rate narrow-waveform units). Note the skewed nature of the distributions in (D) and (E). The extended latencies suggest that many of these cells were activated synaptically via photostimulation of other cells.

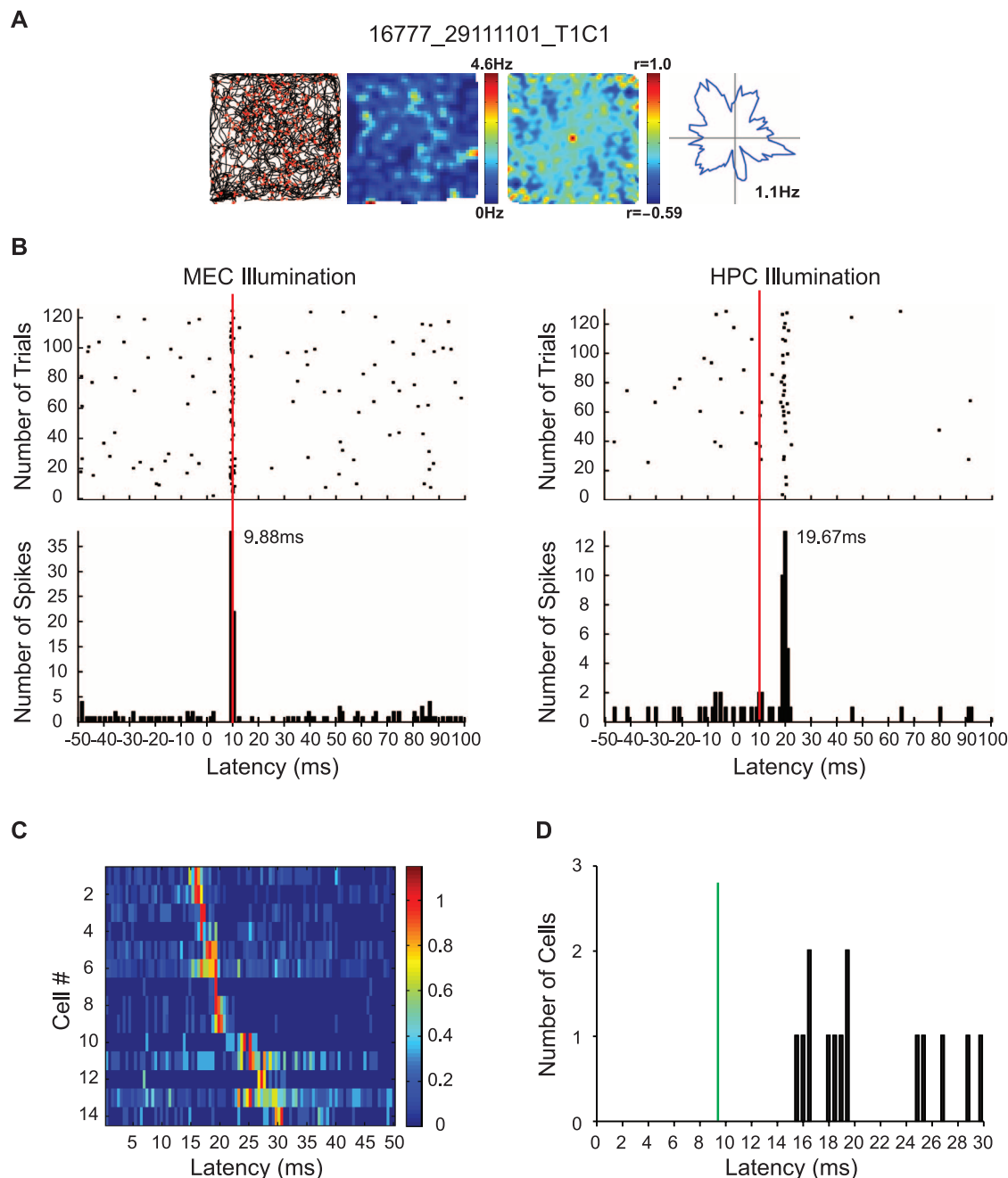


on previous work showing that virus can be transported retrogradely to cell bodies of projection neurons (26, 35, 46), we engineered rAAV to effectively express microbial opsins not only in the infusion area but also in the soma of cells that project to that area, such as the principal cells of

the superficial layers of MEC. Hippocampus-projecting MEC cells could be identified, after expression of ChR2 transgenes, as neurons that responded reliably, at a constant minimal latency, to photostimulation near the cell bodies in the MEC or near axons of these cells in the hippocampus or

the angular bundle. The response latencies of the principal cells were substantially shorter than the latencies of many putative GABAergic MEC neurons, which might have been activated synaptically via photostimulation of ChR2-expressing projection neurons. Response latencies were also

**Fig. 7. Synaptic discharge of MEC neurons after stimulation of hippocampal pyramidal cells.** (A) (Left to right) Trajectory with spike locations, color-coded rate map, spatial autocorrelation map, and directional rate map for a nonspatial cell, as in Fig. 3B. (B) Discharge in the same cell after photostimulation in MEC (left) or stratum pyramidale or stratum oriens of CA1 (right; HPC, hippocampus). Spike rasters and peristimulus spike histograms as in Fig. 3C. The red reference line highlights 10 ms. Note extended spike latency with hippocampal stimulation, which suggests that the cell is activated synaptically from the CA1. (C) Color-coded spike rasters, as in Fig. 4A, showing long latencies when superficial MEC cells were discharged by photostimulation in the CA1 pyramidal cell layer (all cells that responded to superficial CA1 stimulation). (D) Distribution of modal peak latencies across bins of 0.5 ms during the 30 ms after the onset of CA1 stimulation [same sample as in (C)]. Green vertical line indicates modal latency for all principal cells in Fig. 4.



shorter than after stimulation of CA1 outputs from the hippocampus. These differential time courses imply that, in experiments with minimal latencies, the discharge was caused by activation of ChR2 channels in the recorded neurons themselves.

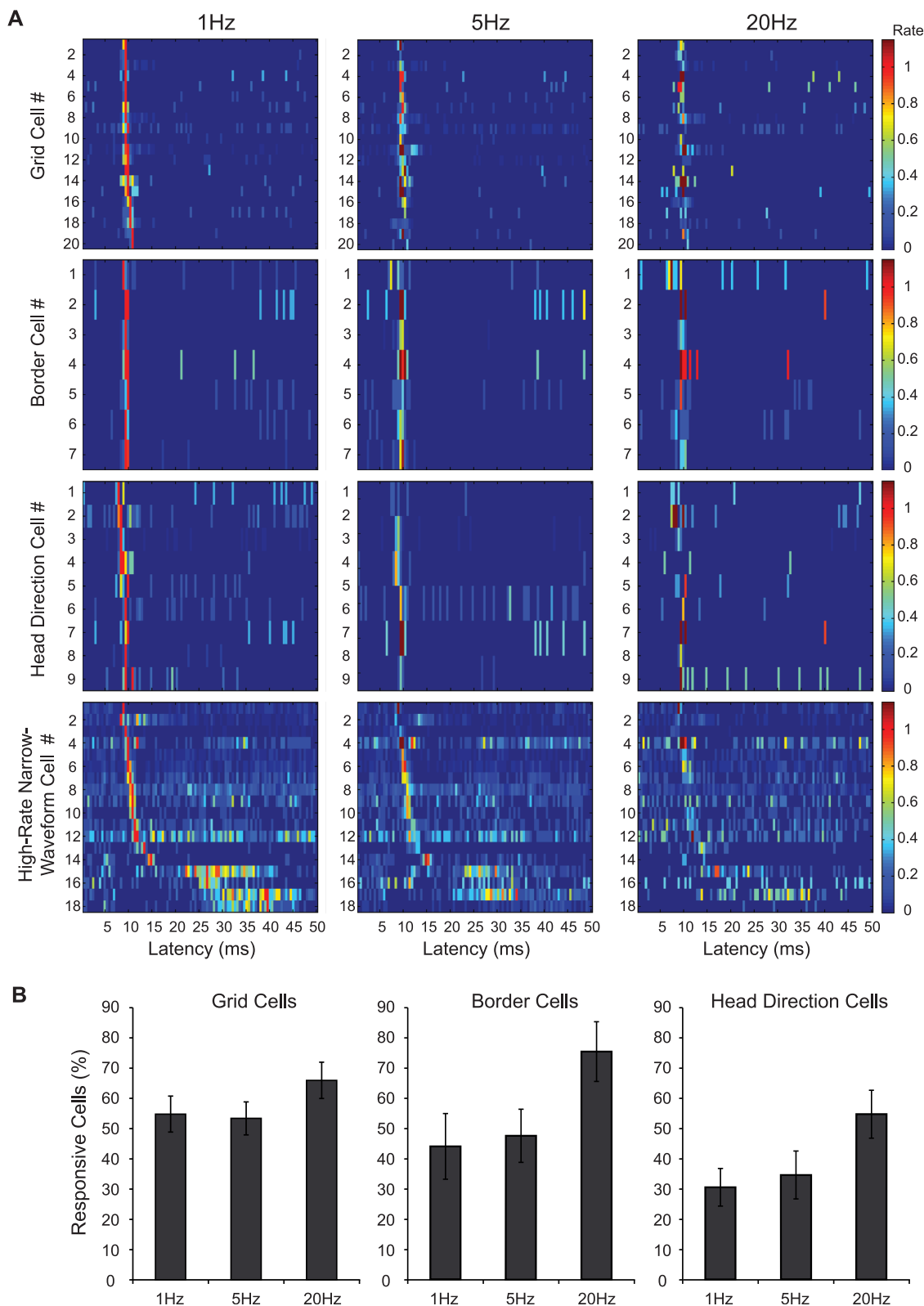
Responsive cells were present in all functional classes of MEC cells, which suggests that the hippocampus receives direct input not only from spatial cells, like grid cells and border cells, but also from head-direction cells, nonperiodic spatial cells, and nonspatial cells. We did not observe any cell type in MEC that did not project to the hippocampus, despite the fact that many principal neurons in layer II have been reported to send axons exclusively to extrahippocampal targets such

as the contralateral MEC (24). The presence of a dual spatial input, from grid cells and border cells, is consistent with the idea that place cells have access to both self-motion and landmark-based information (48) and raises the possibility that the spatial metric of the place-cell population (49, 50) originates from grid cells (17), whereas boundary and landmark-induced firing (37, 38, 51) is derived directly from border cells (18, 19, 52). The dual nature of the spatial input may account for the observation that place cells precede mature grid cells during ontogenesis of the spatial representation system (4, 5) and that place cells can maintain location specificity under conditions that reduce grid-cell periodicity in adult rats (53).

A surprising aspect of the results is that a large component of the MEC input originates from nonspatial cells. If these cells target principal cells, and not only interneurons, they likely provide a major direct-input to the place cell population, considering that nearly all active hippocampal principal cells are place cells (1, 5). The findings thus raise the possibility that individual place cells receive input from a variety of functional cell classes in the MEC. How is then the selective output of the place cells generated? Place cells may have mechanisms for gating particular external inputs (54). These may involve differences in synaptic strength, active dendritic properties, or local circuit mechanisms (22, 23, 55, 56). A similar gating mechanism



**Fig. 8. Light-induced discharge at higher stimulation frequencies. (A)** Color-coded spike rasters showing response to stimulation at 1, 5, and 20 Hz for the entire sample of cells recorded at these frequencies that were responsive at 1 Hz stimulation. Each row shows data for one cell. Time bins are 0.5 ms. Color scale to the right; color is normalized to the firing rate at 1 Hz. For 1 Hz, all light pulses are included. For 5 and 20 Hz, all light pulses except the first in each pulse train are included. (Top) Grid cells; (middle) border cells and head-direction cells; (bottom) high-rate narrow-waveform cells (putative GABAergic neurons). In all three principal cell types, light responses were maintained at the higher frequencies, with similar spike latencies, which suggests that the cells were activated directly rather than via other cells that expressed Chr2. **(B)** Histogram showing percentage of pulses that evoked spikes in grid cells, border cells, and head-direction cells at 1, 5, and 20 Hz photostimulation frequencies (means  $\pm$  SEM). The first spike of the pulse train is not counted. Note that all functional cell types continued to respond at the highest stimulation frequency.



may be present in layer II/III of the visual cortex, where orientation-selective neurons convert widely tuned synaptic inputs to highly specific output signals (57). Convergent input from a broad spectrum of entorhinal cell types would enable individual place cells to respond

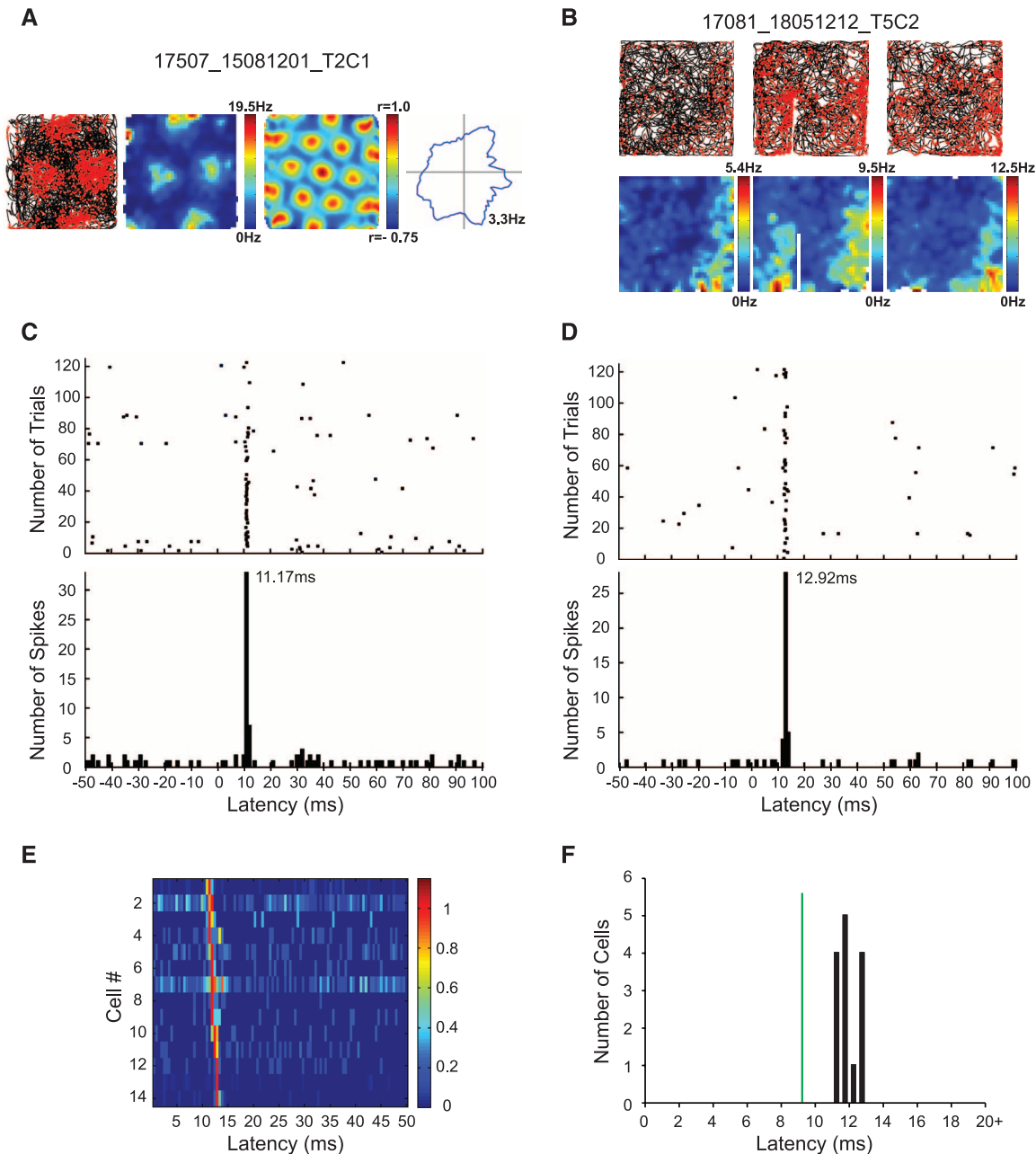
more dynamically, such that different types of input could be favored in different states or under different behavioral circumstances. This may greatly increase the representational and computational capacity of the hippocampal network.

## Materials and Methods

### AAV Vector Constructs

All viral constructs were generated by a polymerase chain reaction (PCR)-based amplification and cloning method. The coding region in all viral

**Fig. 9. Antidromic discharge of MEC neurons after photostimulation of entorhinal projections to the hippocampus.** (A and B) Two example cells that responded to antidromic stimulation. (A) is a grid cell (left to right: trajectory with spike locations, color-coded rate map, directional rate map, and autocorrelation map); (B) is a border cell (top row, trajectory with spike locations; bottom row, rate maps). Insertion of a wall induced a firing field on one side of the wall (second trial from the left), mirroring the field at the peripheral wall on the baseline trial. Symbols as in Fig. 3, B and E. (C and D) Photoinduced antidromic discharge of the cells in (A) and (B), respectively, after photostimulation in the angular bundle (C) or the perforant path (D). Spike rasters and peristimulus spike histogram as in Fig. 3C. Note short firing latencies, only 1 to 2 ms slower than with local entorhinal stimulation and more than 4 ms faster than with synaptic stimulation from the CA1. The slightly longer delay with stimulation in the perforant path than in the angular bundle likely reflects the longer conduction distance. (E) Color-coded spike rasters showing distribution of spike latencies in all MEC cells that could be discharged by photostimulation in the perforant path—termination zone (cells 1 to 10) or the angular bundle (cells 11 to 14). Symbols as in Fig. 4A. (F) Distribution of modal peak latencies across bins of 0.5 ms during



the 20 ms after the onset of antidromic stimulation [same sample as in (E)]. Green vertical line indicates modal latency for all principal cells in Fig. 4.

vector constructs was verified by double-stranded DNA sequencing to make sure that no shifted open reading frame and no undesired point mutation was introduced by PCR. All proviral plasmids used for packaging rAAV were flanked by AAV serotype-2 inverted terminal repeats (ITRs). rAAV2/1 vectors contained a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) and a bovine growth hormone (BGH) polyadenylation signal for enhancing transgene transcription and expression. In all viral expression cassettes, transcriptional regulation was regulated by either a hybrid cytomegalovirus early enhancer–chicken  $\beta$ -actin (CAG) promoter (in

experiments with halorhodopsin or Arch) or a calcium–calmodulin-dependent protein kinase II  $\alpha$  (CaMKII $\alpha$ ) promoter (in experiments with Chr2), which each efficiently drive transduction of rAAV2/1 in both principal cells and interneurons (58). pEYFP-Nuc (Clontech, Mountain View, California, USA) and pcNDA3-LacZ (Invitrogen, Grand Island, New York, USA) were used as PCR templates to generate rAAV-EYFP-Nuc and rAAV-LacZ, respectively. The opsin-expressing rAAV contained either the light-driven cation channel Chr2 or the inward chloride pump halorhodopsin (eNpHR 3.0) or the outward proton pump archaerhodopsin-3 (Arch). For making rAAV

encoding mammalian codon-optimized opsins, pAAV-CaMKII $\alpha$ -hChr2(H134R)-EYFP (gift from Karl Deisseroth, Stanford University, Stanford, California, USA), pAAV-CaMKII $\alpha$ -eNpHR3.0-EYFP (gift from Karl Deisseroth, Stanford University, Stanford, California, USA) and AAV-FLEX-Arch-GFP (GFP, green fluorescent protein) (gift from Edward Boyden, MIT, Cambridge, Massachusetts, USA) were used as PCR templates to generate rAAV-CaMKII $\alpha$ -Chr2, rAAV-CaMKII $\alpha$ -NpHR, and rAAV-CaMKII $\alpha$ -Arch, respectively. For generating trafficking-enhanced opsins, a FLAG tag was placed at the C terminus of all opsin genes between a 20–amino acid trafficking sig-



nal DYKDHDGDYKDHDIDYKDDDDK and an endoplasmic reticulum (ER)-exporting motif FCYENEV, both derived from the inward-rectifier potassium ion channel Kir2.1 and introduced to improve membrane trafficking (34, 35). The 17-amino acid N-terminal signal peptide from the  $\beta$  subunit of nicotinic acetylcholine receptor (nAChR) originally used for membrane insertion in eNpHR2.0 (35) was also removed. This resulted in the creation of trafficking-enhanced microbial opsins to be used for photostimulation.

### rAAV Preparation and Titering

Chimeric rAAV2/1 was serotyped with AAV1 capsid proteins. The pseudotyped rAAV2/1 was prepared by cotransfection of human embryonic kidney cell line HEK293 by standard calcium phosphate transfection along with an adenoviral helper plasmid pHelper from Stratagene's AAV Helper-Free System (La Jolla, California, USA). Twelve hours after transient transfection, the DNA/CaCl<sub>2</sub> mixture was replaced with normal growth medium. After an additional 60 hours in culture, the transfected cells were collected and subjected to three freeze/thaw cycles. The clear supernatant was then purified using heparin affinity columns (HiTrap Heparin HP, GE Healthcare, Uppsala, Sweden). The purified rAAV2/1 was concentrated in an Amicon Ultra-4 centrifugal filter 100K device (Millipore, Billerica, Massachusetts, USA). Viral titers of all prepared rAAV2/1 were determined by real-time quantitative PCR using StepOnePlus Real-Time PCR Systems (Applied Biosystems, Foster City, California, USA) and TaqMan Universal Master Mix as previously described (59). All titered viruses were diluted and matched to  $1.0 \times 10^{12}$  viral genomic particles/ml by  $1 \times$  phosphate-buffered saline (PBS).

### Subjects

Neuronal activity was recorded from MEC in 34 male Long-Evans rats (3 to 5 months old, 350 to 450 g at surgery). The animals were housed individually in transparent Plexiglas cages (45  $\times$  30  $\times$  35 cm) in a temperature- and humidity-controlled vivarium 5 to 10 m from the recording rooms. All rats were maintained on a 12-hour light/12-hour dark schedule. Testing occurred in the dark phase. The rats were kept at 85 to 90% of free-feeding body weight and deprived of feed 18 to 24 hours before each training and recording trial. Body weight at the time of surgery was 350 to 400 g. Water was available ad libitum.

### Surgery

On the day of surgery, the rats were anesthetized with isoflurane (induction chamber level of 5.0% with an air flow at 1400 ml/min, gradually reduced after the rats were secured in the stereotaxic apparatus to 1% isoflurane with an air flow at 1000 to 1200 ml/min). Levels of anesthesia were monitored regularly by testing toe and tail pinch reflexes. High titer-matched solution of rAAV was injected into the hippocampus over a period of 5 to 10 min at three

locations within the dorsal hippocampus (AP: 4.1 mm; ML:  $\pm$ 2.6 mm; DV: 3.5, 2.8, and 2.1 mm), using a 10- $\mu$ l NanoFil syringe (World Precision Instruments, Sarasota, Florida, USA) and a 33-gauge beveled metal needle. Injection volume (0.5 to 1  $\mu$ l at each location) and flow rate (0.1  $\mu$ l/min) were controlled with a Micro4 Microsyringe Pump Controller (World Precision Instruments). After injection, the needle was left in place for 10 additional minutes before it was withdrawn slowly. During the same surgical session, after hippocampal rAAV injection, the animals were implanted with tetrodes and optic fibers in the MEC and the hippocampus.

All animals were implanted with microdrives in MEC, which each were connected to an assembly of four tetrodes, cut flat at the same level, as well as a 125- $\mu$ m wide optic fiber with the tip 500  $\mu$ m above the tetrode tips. The tetrode-fiber assembly was implanted 0.1 to 0.5 mm in front of the transverse sinus, 4.5 to 4.7 mm from the midline, and 1.6 to 1.8 mm below the dura. Implants were oriented at an 8 to 20 degree angle in the anterior direction in the sagittal plane. Five of the animals had an additional optic fiber implanted in the ipsilateral pyramidal cell layer (or stratum oriens or alveus) of dorsal CA1 (3.8 mm behind bregma, 3.0 mm lateral to the midline, 1.8 mm below dura). Five other animals had an optic fiber in the perforant-path termination zone of the dorsal hippocampus (perforant path: 4.3 mm behind bregma, 2.4 mm lateral to the midline,  $\sim$ 2.6 mm below dura; angular bundle: 7.8 mm behind bregma, 4.2 mm lateral to the midline,  $\sim$ 2.8 mm below dura), in addition to tetrodes and optic fiber in the MEC. In three of the animals, cells were also recorded with tetrodes in CA3 in response to local stimulation in this region. Three of the animals had a 26-ga cannula (C315G; Plastics One, Roanoke, Virginia, USA) in the MEC, near the tetrode-fiber assembly. Finally, two animals with fibers and tetrodes in MEC received rAAV infusions in the cortex overlying the hippocampus.

The tetrodes were made of 17- $\mu$ m polyimide-coated platinum-iridium (90 to 10%) wire. The electrode tips were platinum-plated to reduce electrode impedances to  $\sim$ 200 k $\Omega$  at 1 kHz. A jeweler's screw fixed to the skull served as a ground electrode. The microdrives were secured to the skull using additional jewelers' screws and dental cement. After surgery, the rats were allowed 1 week of recovery before handling and/or habituation to the test environments was resumed.

### Training and Neuronal Recording Procedures

Tetrode turning started 2 to 3 days after viral infection and implantation, but data collection from MEC did not start until 2 to 4 weeks later. Before each recording trial, the rat rested on a towel in a large flower pot on a pedestal. The rat was connected to the recording equipment via AC-coupled unity-gain operational amplifiers close to the rat's head, using a counterbalanced cable that allowed the animal to move freely in

the pot and the recording boxes. Over the course of 10 to 20 days, the tetrodes were lowered in steps of 50  $\mu$ m or less until single neurons could be isolated at appropriate depths. When the signal amplitudes exceeded  $\sim$ four times the noise level (root mean square 20 to 30  $\mu$ V) and the units were stable for more than 1 hour, data were recorded. After each finished set of experiments, the tetrodes were moved further until new well-separated cells were encountered.

Recorded signals were amplified 8000 to 25,000 times and band-pass filtered between 0.8 and 6.7 kHz. Triggered spikes were stored to disk at 48 kHz (50 samples per waveform, 8 bits/sample) with a 32-bit time stamp (clock rate at 96 kHz). An electroencephalogram (EEG) was recorded single-ended from one of the electrodes. The EEG was amplified 3000 to 10,000 times, low pass-filtered at 500 Hz, sampled at 4800 Hz, and stored with the unit data. By means of an overhead video camera, the recording system tracked the position of two light-emitting diodes (LEDs), one large and one small, on the head stage (sampling rate 50 Hz). The LEDs were separated by 5 to 10 cm and aligned with the body axis of the rat.

In parallel with the turning of the tetrodes, over the course of 3 to 6 weeks, the animals were trained to run around in a square black aluminum enclosure polarized by a white cue card. Running was motivated by randomly scattering crumbs of chocolate at 10- to 15-s intervals in the recording enclosure. Each trial lasted 10 or 15 min.

In rats with putative border cells, the recording trial in the square box was succeeded by a test in the same box in which a separate wall (50 cm long  $\times$  50 cm high) was inserted between the center of one of the external walls and the center of the box. This test was followed by another trial without the wall. These trials were 10 min each.

### Recording of Light-Responsive Neurons

Identification of ChR2-expressing functional cell types was performed by first recording neural activity while the rat ran for 10 or 15 min in the square black aluminum enclosure. The functional identity of cells recorded during this trial was determined by estimating the spatial periodicity, the spatial information values, the directional modulation, and the proximity to borders of each cell's neural firing pattern and then comparing these values to shuffled data from the entire population of recorded neurons, as described below.

After the recording in the square enclosure, the rat was moved back to the pot and, after 2 min of baseline recording during rest, the photostimulation started. In the main experiment, photostimulation was delivered into MEC, above the tetrode tips. The stimulation consisted of 3.5-ms 473-nm light pulses delivered repeatedly at a power density of 10 mW/mm<sup>2</sup> for one or three periods of 2 min. All animals received stimulation at a frequency of 1 Hz for 2 min. Sixteen of the animals received additional blocks of

stimulation with light pulses delivered at 5 and 20 Hz, respectively, in blocks of 5 pulses for 5 Hz and 10 pulses for 20 Hz, at intervals of 5 s for a duration of 2 min each. In a subset of experiments, photostimulation was delivered in the CA1 region of the hippocampus, in the perforant path–termination zone at a deeper location, or in the angular bundle posterior to the hippocampus. Virally transduced cells were in all cases identified as cells that fired reliably at fixed latencies in response to the photostimulation.

To validate the interpretation that the short latency–activated neurons were directly stimulated, we tested whether firing could be induced optically after blockade of excitatory neurotransmission in the recording area. Three rats were implanted with cannulae above the fiber-tetrode bundle in the MEC. Once light-responsive neurons were identified in MEC, a cocktail of the competitive AMPA/kainate receptor antagonist CNQX and the competitive NMDA receptor antagonist D-APV was infused to interrupt glutamatergic neurotransmission at the recording location. CNQX-APV cocktail (60), dissolved in sterile 0.9% saline (3 mM for CNQX and 30 mM for APV) and with final pH adjusted to 7.2, was infused via a 33-ga internal cannula (C315I; Plastics One, Roanoke, Virginia, USA) connected by polyethylene tubing to a 25- $\mu$ l syringe mounted in a CMA/100 infusion pump. The tip of the inner cannula protruded 0.9 mm beyond the implanted guide cannula. The infusion rate (0.1  $\mu$ l/min) was controlled by the syringe pump. The total volume of each infusion was 0.5 to 2.0  $\mu$ l. The inner cannula was retracted 10 min after the infusion. After the infusion, recording in the flower pot was resumed.

### Spike Sorting, Cell Classification, and Rate Maps

Spike sorting was performed offline using graphical cluster-cutting software (fig. S13). Position estimates were based on tracking of one of the LEDs on the head stage. Only epochs with instantaneous running speeds of 2.5 cm/s or more were included. To characterize firing fields, the position data were sorted into 2.5-cm  $\times$  2.5-cm bins, and the path was smoothed with a 21-sample boxcar window filter (400 ms; 10 samples on each side) (11). Maps for number of spikes and time were smoothed individually using a quasi-Gaussian kernel over the surrounding 5  $\times$  5 bins (11). Firing rates were determined by dividing spike number and time for each bin of the two smoothed maps. The peak rate was defined as the rate in the bin with the highest rate in the firing rate map.

### Identification of Photoresponsive Cells

Photoexcitable cells were formally identified by comparing firing rates as a function of stimulation latency during the 100 ms after each light pulse with the firing rates obtained for similar time blocks after shuffling the spike times of each cell within an interval [–100, 100 ms] around the light stimulus. After each shuffling, spikes were counted for bins of 1 ms after the stimulation,

and the three successive bins with the maximum number of spikes during the 100 ms period after the stimulation were identified. A similar block of three successive bins was identified for the real data. For each cell, the spike times were shuffled 10,000 times. Cells were classified as photoreponsive if the number of spikes in the block with maximal number of spikes in the real data exceeded the 99.9th percentile value of the distribution of number of spikes in the most active triple for the shuffled data. The latency of the response was taken as the mean latency of all spikes contributing to this 3-bin block.

### Analysis of Grid Cells

The structure of the rate maps was evaluated for all cells with more than 100 spikes by calculating the spatial autocorrelation for each smoothed rate map (10). Autocorrelograms were based on Pearson's product moment correlation coefficient with corrections for edge effects and unvisited locations. With  $\lambda(x, y)$  denoting the average rate of a cell at location  $(x, y)$ , the autocorrelation between the fields with spatial lags of  $\tau_x$  and  $\tau_y$  was estimated as:

$$r(\tau_x, \tau_y) = \frac{n \sum \lambda(x, y) \lambda(x - \tau_x, y - \tau_y) - \sum \lambda(x, y) \sum \lambda(x - \tau_x, y - \tau_y)}{\sqrt{n \sum \lambda(x, y)^2 - [\sum \lambda(x, y)]^2} \sqrt{n \sum \lambda(x - \tau_x, y - \tau_y)^2 - [\sum \lambda(x - \tau_x, y - \tau_y)]^2}}$$

where the summation is over all  $n$  pixels in  $\lambda(x, y)$  for which rate was estimated for both  $\lambda(x, y)$  and  $\lambda(x - \tau_x, y - \tau_y)$ . Autocorrelations were not estimated for lags of  $\tau_x, \tau_y$  where  $n < 20$ .

The degree of spatial periodicity (“gridness” or “grid scores”) was determined for each recorded cell by taking a circular sample of the autocorrelogram, centered on the central peak but with the central peak excluded, and comparing rotated versions of this sample (5, 10, 11). Gridness (the cell's grid score) was defined as the minimum difference between any of the elements in the first group and any of the elements in the second. Grid cells were defined as cells in which rotational symmetry–based grid scores exceeded the 99th percentile of a distribution of grid scores for shuffled recordings from the entire population of MEC cells. Shuffling was performed by time-shifting, for each permutation trial, the entire sequence of spikes fired by the cell along the animal's path by a random interval between 20 s and 20 s less than the length of the trial (usually 600 – 20 s = 580 s), with the end of the trial wrapped to the beginning. The shuffling procedure was repeated 100 times for each of the 779 recordings in the photoexcitation study and each of 270 cells in the photoinhibition study, yielding a total of 77,900 and 27,000 permutations, respectively. For each permutation, a firing rate map and an autocorrelation map were constructed, and a grid score was calculated. The 99th percentile was then read out from the overall distribution of grid scores. Grid cells were defined as cells with grid scores higher than the 99th per-

centile of the grid scores of the distribution for the shuffled data (fig. S9A).

### Analysis of Head-Direction Cells

The rat's head direction was calculated for each tracker sample from the projection of the relative position of the two LEDs onto the horizontal plane. The directional tuning function for each cell was obtained by plotting the firing rate as a function of the rat's directional heading, divided into bins of 3 degrees and smoothed with a 15 degrees mean window filter (two bins on each side) (4, 5, 11). In order to minimize the contribution of inhomogeneous sampling on directional tuning estimates, data were accepted only if all directional bins were covered by the animal.

The strength of directional tuning was estimated by computing the length of the mean vector for the circular distribution of firing rate (5, 11). Head direction–modulated cells were defined as cells with mean vector lengths significantly exceeding the degree of directional tuning that would be expected by chance for the MEC population. Threshold values were determined by a shuffling procedure performed in the same way as for grid cells. For each permutation trial, the entire sequence of spikes fired by the cell was time-shifted along the animal's path by a random interval between on one side 20 s and on the other side 20 s less than the length of the trial, with the end of the trial wrapped to the beginning, a head-direction tuning curve was then constructed, and the mean vector length was calculated. The distribution of mean vector lengths was computed for the entire set of permutations from all cells in the sample (77,900 permutations for the photoexcitation study, 27,000 permutations for the photoinhibition study, and 100 permutations per cell). Cells were defined as directionally modulated if the mean vector from the recorded data was longer than the 99th percentile of mean vector lengths in the distribution generated from the shuffled data (fig. S11A).

### Analysis of Border Cells

Border cells were identified by computing, for each cell, the difference between the maximal length of a wall touching upon any single firing field of the cell and the average distance of the field from the nearest wall, divided by the sum of those values (11, 12). Firing fields were defined as collections of neighboring pixels with firing rates higher than 0.3 times the cell's peak firing rate that covered a total area of at least 200 cm<sup>2</sup>. Border scores ranged from –1 for cells with central firing fields to +1 for cells with fields that perfectly line up along at least one entire wall. Border cells were defined as cells with border scores significantly exceeding the degree of wall-related firing that would be expected for MEC cells by chance. The significance level was determined by a shuffling procedure performed for experiments in the square boxes in the same way as for grid cells and head-direction cells. For each permutation trial, the entire sequence of spikes fired by the cell was time-shifted along the



animal's path by a random interval between 20 s and 20 s less than the length of the trial, with the end of the trial wrapped to the beginning, a rate map was then constructed, and a border score was calculated. The distribution of border scores was computed for the entire set of permutations from all cells in the sample (77,900 permutations for the photoexcitation study, 27,000 permutations for the photoinhibition study, and 100 permutations per cell), and the 99th percentile was determined. Cells were defined as border cells if the border score from the recorded data was higher than the 99th percentile for border scores in the distribution generated from the shuffled data (fig. S10A).

### Analysis of Irregular Spatial Cells and Nonspatial Cells

Spatially modulated cells with irregular firing fields were defined as cells that did not satisfy criteria for grid cells, border cells, or head-direction cells but had spatial information values that exceeded the 99th percentile of the shuffled data (fig. S12A). Nonspatial cells were defined as cells that satisfied neither of the above criteria.

For each cell, the spatial information content in bits per spike was calculated as

$$\text{information content} = \sum_i p_i \log_2 \frac{\lambda_i}{\lambda}$$

where  $\lambda_i$  is the mean firing rate of a unit in the  $i$ th bin,  $\lambda$  is the overall mean firing rate, and  $p_i$  is the probability of the animal being in the  $i$ th bin [(occupancy in the  $i$ th bin)/(total recording time)] (61).

An adaptive smoothing method (62) was used before the calculation of information scores in order to optimize the trade-off between blurring error and sampling error. The raw data were first divided into bins of 2.5 cm  $\times$  2.5 cm. Then, the firing rate at each point in the environment was estimated by expanding a circle around the point until

$$r \geq \frac{\alpha_i}{n\sqrt{s}}$$

where  $r$  is the radius of the circle in bins,  $n$  is the number of occupancy samples within the circle,  $s$  is the total number of spikes in those occupancy samples, and the constant  $\alpha$  is set to 10,000. With a position sampling rate of 50 Hz, the firing rate at that point was then set to 50  $\cdot$   $s/n$ .

The chance level for spatial information was determined by the same random permutation procedure as used for the other cell types. Spatial cells were defined as cells in which spatial information values exceeded the 99th percentile of a distribution of such values for shuffled recordings from the entire population of MEC cells. Shuffling was performed by time-shifting, for each permutation trial, the entire sequence of spikes fired by the cell along the animal's path by a random interval between 20 s and 20 s less than the length of the trial (usually 600 – 20 = 580 s), with the end of the trial wrapped to the beginning. The shuffling procedure was repeated 100 times for each of

the 726 recordings in the photoexcitation study, yielding a total of 72,600 permutations. For each permutation, the spatial information value of the firing rate distribution was determined. The 99th percentile was then read out from the overall distribution and used as a threshold to define spatial (but nonperiodic) MEC cells.

### Histology, Immunohistochemistry, and Reconstruction of Recording Positions

Electrodes were not moved after the final recording session. The rats received an overdose of sodium pentobarbital and were transcardially perfused with 0.9% saline followed by 4% formaldehyde. The perfused brain with skull and microdrive was soaked into 4% formaldehyde for 2 to 4 hours, the electrodes were turned all the way up, and the brain was extracted and stored in 4% formaldehyde. At least 24 hours later, the brains were quickly frozen and cut by a Micron Cryo-Star HM560 Cryostat (Micron International, Germany) at 30  $\mu$ m in the sagittal plane. All sections around the area of the tetrode trace were collected and mounted on glass. For every pair of consecutive sections, the first was stained with cresyl violet (Nissl) and the second was assigned for staining by antibodies. The latter group of sections was divided into six interleaved sets by placing the sections sequentially into a row of 6 wells in a 24-well plate containing 1 $\times$  PBS, such that each section set could be used against different antibodies (FLAG, NeuN, or GFP).

For the immunostaining, sections were rinsed three times for 10 min in 1 $\times$  PBS at room temperature and preincubated for 2 hours in 10% normal goat serum in PBST (1 $\times$  PBS with 0.5% Triton X-100). All rinses between incubation steps were with PBST. After rinsing, processed sections were incubated with different primary antibodies against FLAG (Sigma, 1:2000), NeuN (Millipore, 1:500), GFP (Clontech, 1:2000), c-Fos (Calbiochem, 1:2000), and glial fibrillary acidic protein (GFAP) (Sigma, 1:1000) for 72 hours in antibody-blocking buffer at 4°C. After three times of 15-min washing in 1 $\times$  PBST at room temperature, sections were incubated either in a mouse antibody directed against a donkey secondary antibody or a donkey antibody directed against a rabbit secondary antibody conjugated with either fluorescein isothiocyanate or Cy3, respectively (Jackson ImmunoResearch, West Grove, Pennsylvania, USA, 1:2000), for 2 hours at room temperature. After intensive rinse with 1 $\times$  PBST, sections were mounted onto glass slides with 4',6'-diamidino-2-phenylindole (DAPI)-containing Vectashield mounting medium (Vector Laboratories, Burlingame, California, USA), and a cover slip was applied. Expression of microbial opsins was estimated by anti-FLAG antibody, and the C-terminal FLAG tag was fused with the opsin in the viral construct. NeuN was used for staining neuronal cell types only. GFP was used for rAAV-EYFP-Nuc infected neurons.

The positions of the tips of the recording electrodes were determined from digital pictures of the

Nissl-stained sections. Scanning and measurements were made using MIRAX MIDI software (Carl Zeiss, Germany). A shrinkage coefficient was calculated by measuring the distance (on the digital image) from the surface of the brain to the tips of the recording electrodes and then dividing this by the final depth of the electrodes, read out from the turning protocol. Only recordings obtained in the superficial layers of MEC or at the MEC-parasubiculum border were analyzed.

For rats infused with rAAV-LacZ,  $\beta$ -galactosidase activity was also carried out in parallel with anti-FLAG immunostaining. Briefly, sections were first fixed for 2 hours with 4% paraformaldehyde in 1 $\times$  PBS and then washed with 100 mM phosphate buffer (pH 7.4), 2 mM MgCl<sub>2</sub>, and 5 mM EGTA (pH 8.0) for four times of 15 min each at room temperature. To develop blue precipitates, sections were then immersed into X-gal staining solution with 100 mM phosphate buffer (pH 7.4), 2 mM MgCl<sub>2</sub>, 0.01% sodium desoxycholate, 0.02% Nonidet P-40, 5 mM potassium-ferri-cyanide, 5 mM potassium-ferrocyanide, and 0.5 mg/ml of X-gal in the dark at 37°C with a humidified chamber under gentle shaking. Once the blue color was developed to a maximum, X-gal staining was stopped by rinsing sections with 1 $\times$  PBS.

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# Supplementary Materials

[www.sciencemag.org/cgi/content/full/science.1232627/DC1](http://www.sciencemag.org/cgi/content/full/science.1232627/DC1)  
Figs. S1 to S21  
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# An Integral View of Fast Shocks Around Supernova 1006

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Supernova remnants are among the most spectacular examples of astrophysical pistons in our cosmic neighborhood. The gas expelled by the supernova explosion is launched with velocities ~1000 kilometers per second into the ambient, tenuous interstellar medium, producing shocks that excite hydrogen lines. We have used an optical integral-field spectrograph to obtain high-resolution spatial-spectral maps that allow us to study in detail the shocks in the northwestern rim of supernova 1006. The two-component H $\alpha$  line is detected at 133 sky locations. Variations in the broad line widths and the broad-to-narrow line intensity ratios across tens of atomic mean free paths suggest the presence of suprathermal protons, the potential seed particles for generating high-energy cosmic rays.

Supernova remnants, the expanding shells of material created in a stellar explosion, are astrophysical laboratories for studying nonthermal physics and high-velocity shocks and have been scrutinized over a broad range of wavelengths. The signatures of nonthermal electrons are typically manifested in the x-ray,  $\gamma$ -ray, and radio range of wavelengths (1). Complementary to these observations, hydrogen emission from Balmer-dominated shocks (2–4), around supernova remnants of thermonuclear origin, directly probe the proton populations (5). Until now, studies of Balmer-dominated shocks have typically used conventional spectrographs that yield rich spectral information but limited spatial information. Here, we report on Balmer-dominated shocks around a supernova remnant by using integral-field unit (IFU) spectroscopy, a technique that produces a three-dimensional “data cube”: two dimensions of space (across the sky) and a spectral dimension. We selected supernova (SN) 1006 as our target, because it has a long history of serving as a laboratory for studying nonthermal physics and high-velocity shocks (6–12).

Long-slit spectroscopy, used in previous studies of Balmer-dominated shocks (13), cumulatively measures the H $\alpha$  emission emanating from regions much larger than the characteristic length scale: the mean free path for interactions between hydrogen atoms and electrons or ions,  $L_{\text{mfp}} \sim 1/n\sigma_{\text{ce}}$  (with  $n$  being the preshock num-

ber density and  $\sigma_{\text{ce}} \sim 10^{-15} \text{ cm}^2$  denoting the typical cross section for charge exchange). With use of the inferred range of densities for the northwestern rim of SN 1006 of  $n$  from 0.15 to  $0.40 \text{ cm}^{-3}$  (14–16), we estimated that the  $0.67''$  size of a pixel in our IFU observations corresponds to  $\sim 5L_{\text{mfp}}$ . The high spatial resolution allowed us to separate out the contributions of bulk motion versus thermal velocity to the measured line widths, an issue that has limited the interpretation of previous observations. It also enabled us to study the spatial variation of the line widths and ratios and hence changes in microphysics of a shock across several atomic mean free paths. To buttress the second point, we have intentionally chosen a field of view that zooms in on a region of simple geometry, implying that any substantial spatial variation detected cannot be due to variations in density caused by geometric or projection effects.

Figure 1 illustrates the general configuration of our observations performed with VIMOS (Visible Multi-Object Spectrograph) in the IFU mode on the Very Large Telescope. We have focused on the northwestern rim, because it produces the brightest emission from a Balmer-dominated shock. As a first analysis of our data, we divided the shock structure into four strips and binned up the data along each strip. The two-component H $\alpha$  line is convincingly detected. To first order, the broad line full width at half maximum (FWHM)  $W$  yields the shock velocity  $v_s$ , whereas the level of energy equilibration between electrons and protons in the postshock gas introduces small corrections (17, 18). Because the broad line profile is a direct probe of the velocity distribution of the postshock protons and the narrow line profile traces preshock hydrogen atoms, the ratio of broad to narrow line intensities,  $I_b/I_n$ , contains information on how energy is shared between the pre- and postshock regions. If no energy is shared, models that include the basic shock and atomic

physics correctly translate the measured  $W$  and  $I_b/I_n$  values into the inferred  $v_s$  and  $\beta$  values (13, 19–21), where  $\beta$  is the ratio of electron to proton temperatures in the postshock gas. Unusually low  $I_b/I_n$  values ( $\lesssim 0.7$ ) indicate that suprathermal particles from the postshock region are traveling upstream into the preshock gas and depositing energy via atomic interactions (excitation, ionization, and charge exchange), thus acting as precursors (22–24). The loss of energy decreases the shock velocity and hence  $W$ . The increased flux of particles into the preshock region leads to enhanced excitation of the hydrogen atoms via collisions and increases  $I_n$ . The binned regions in Fig. 1 already hint at this phenomenon, because  $I_b/I_n \approx 0.6$  to  $0.7$  in two of the strips (yellow and red). The broad line exhibits subtle deviations from a Maxwellian profile in the line core, further supporting the presence of suprathermal particles in the shock.

We refined our approach by using the technique of Voronoi binning (25). We combined neighboring spectra into spatial Voronoi bins until a signal/noise ( $S/N$ ) ratio of about 40 was reached (Fig. 2, bottom right). We fitted double-Gaussians to the detected H $\alpha$  lines as shown in section 2 of the supplementary materials and derived  $W$  and  $I_b/I_n$  values (Fig. 2, top left and top middle). In addition to the lack of complex density variations, we demonstrated that the viewing geometry of the shock is simple: By measuring the velocity shift  $\Delta V$  between the centroids of the narrow and broad H $\alpha$  line components, we derived the shock inclination angle  $\theta$  via the relation  $\Delta V = 3v_s \cos \theta / 4$ . Our measurements indicate that  $\theta \approx 80^\circ$  to  $90^\circ$ . Therefore, the shocks we observed are mostly edge-on as expected, but the rim seems slightly S-shaped along the line of sight as suggested by (14).

We detected significant spatial variations in  $W$  and  $I_b/I_n$  across length scales  $10'' \sim 70L_{\text{mfp}}$  despite the simple geometry of the shocks, suggesting that they arise from variations in the microphysics rather than density. Within the bright rim, the variations in  $W$  are of order 10 to 20%, substantially larger than the individual measurement uncertainties (supplementary materials section 2 and table S1). The density variations of 20 to 40% required to explain the detected variations in  $W$  are much larger than expected on these scales and also incompatible with the unchanged smoothness of the shock over two decades of imaging observations (9, 14).

The low  $I_b/I_n$  values are found at all distances from the inner rim, reaching as low as 0.4 at some locations (Fig. 3). We used the models without nonthermal physics to infer the values of  $v_s$  and  $\beta$  (19). Most (about 85%) of the binned data are not accounted for by the model because of the low  $I_b/I_n$  values, thus motivating the need for models that include suprathermal particles and cosmic rays (26). Combining the derived shock velocities with the proper motion measurement from (9), we obtained estimates of

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the heliocentric distance to SN 1006. Clearly, there has to be a unique distance, likely given by the upper points around  $\sim 2$  kpc (supplementary materials section 3). However, most points underestimate the distance, because loss of energy from the broad line component results in too low inferred shock velocities.

These low values and variations of  $W$  and  $I_b/I_n$ , as well as the potential non-Gaussian contributions to the broad H $\alpha$  lines, demand an explanation. We examined three possibilities. The first is that “broad neutrals” are acting as a precursor (27, 28). These are secondary populations of “hot” hydrogen atoms that are produced when postshock protons capture an electron from a preshock hydrogen atom—the subsequent excitation of these broad neutrals produces the broad H $\alpha$  line component detected in our observations (2–4, 20). Broad neutrals will warm

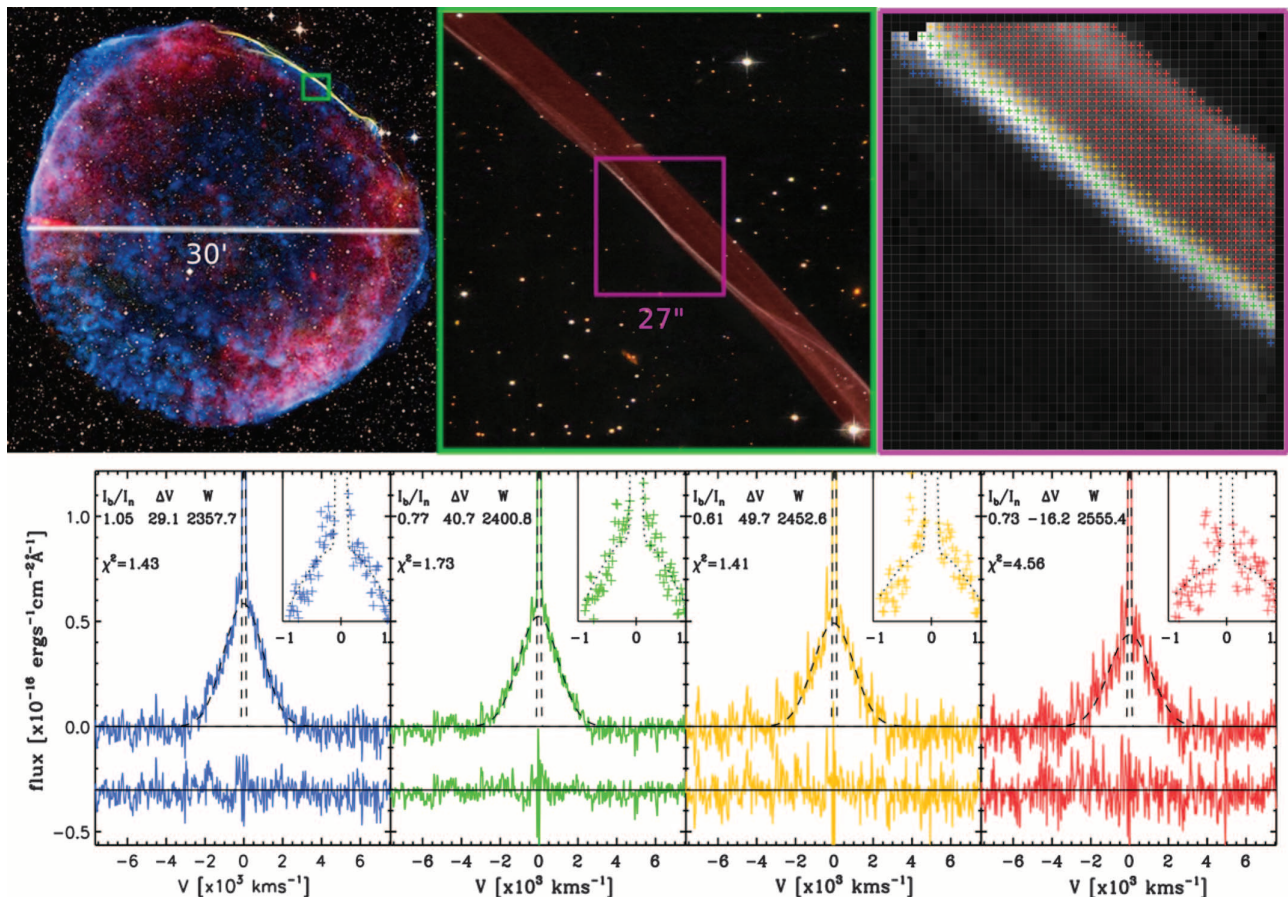
the preshock gas and produce a third H $\alpha$  line component of intermediate width, which may account for the narrow-plus-broad-line double-Gaussian being unable to fit the data within the measured uncertainties (i.e., not reaching a reduced  $\chi^2$  of unity). The strongest argument against broad neutrals is that they can only act as a precursor over an atomic mean free path, which is at odds with our observational result that the variations and low values of  $W$  and  $I_b/I_n$  extend over distances  $\gg L_{\text{mfp}}$ .

The second possibility is that preshock hydrogen atoms may cross the shock front, eventually become ionized, and become protons gyrating along an ambient magnetic field line. These “pick-up protons” settle into a bispherical distribution that introduces a non-Gaussian contribution to the broad H $\alpha$  line core (29). This explanation requires the magnetic field to be ordered on small

length scales ( $\ll L_{\text{mfp}}$ ), whereas turbulent magnetic fields will result in a broad H $\alpha$  line that is approximately Gaussian.

Both explanations are disfavored at low preshock neutral fractions, which is the situation in SN 1006, with a preshock neutral fraction of about 0.1 (13). This is because both broad neutrals and pick-up ions require the preshock gas to contain a substantial population of hydrogen atoms (relative to electrons and protons) in order to initiate the process. In the limit of a fully ionized preshock gas, no broad neutrals or pick-up ions may be produced.

The explanation we favor is that the postshock proton population includes a nonthermal subpopulation of protons: suprathermal protons (that are not pick-up protons). Such an explanation requires no assumption on the magnetic field geometry or preshock neutral fraction. The

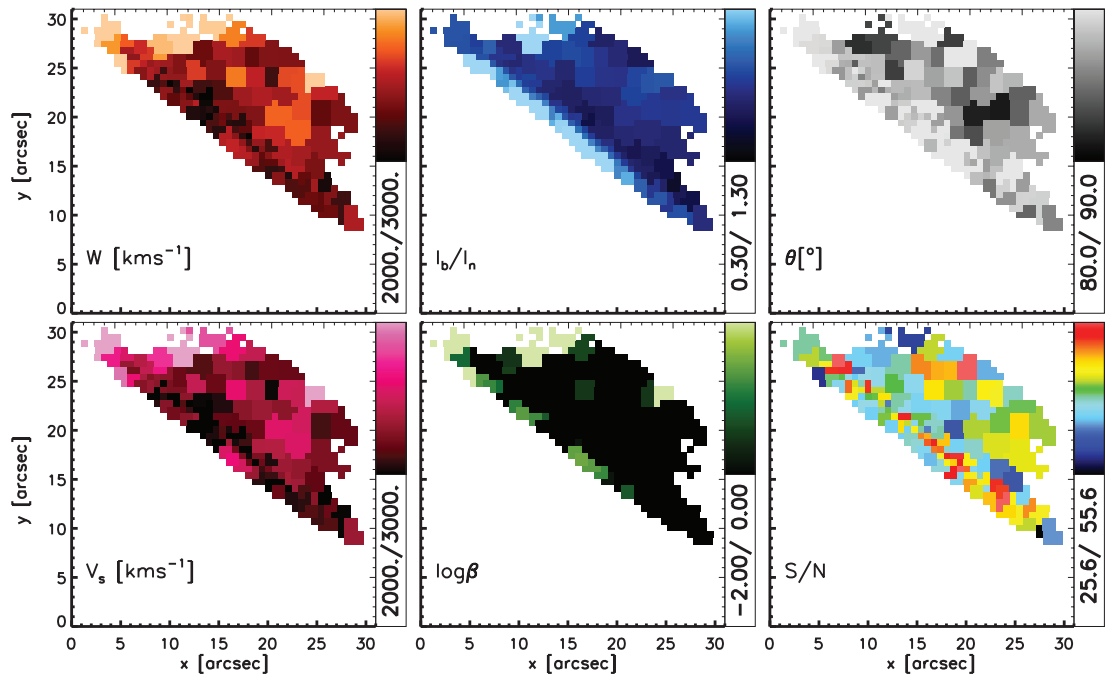


**Fig. 1. VIMOS-IFU spectroscopy of the shock front in the remnant of SN 1006.** (Top left) A composite image of the full remnant ( $\approx 30'$  in diameter), combining data from the Very Large Array and Green Bank Telescope (red) [National Radio Astronomy Observatory/Associated Universities, Incorporated/National Science Foundation (NSF)/K. K. Dyer, R. J. Maddalena, and T. J. Cornwell], Chandra X-ray Observatory (blue) (NASA/Rutgers/G. Cassam-Chenaï *et al.*), 0.9-m Curtis Schmidt optical telescope (yellow) (National Optical Astronomy Observatory/Association of Universities for Research in Astronomy/NSF/Cerro Tololo Inter-American Observatory/Middlebury College/F. Winkler), and Digitized Sky Survey (orange and light blue stars). The green box indicates the region covered by the Hubble Space Telescope H $\alpha$  narrow-band image (top middle), with the magenta box indicating the region observed with the VIMOS-IFU. (Top

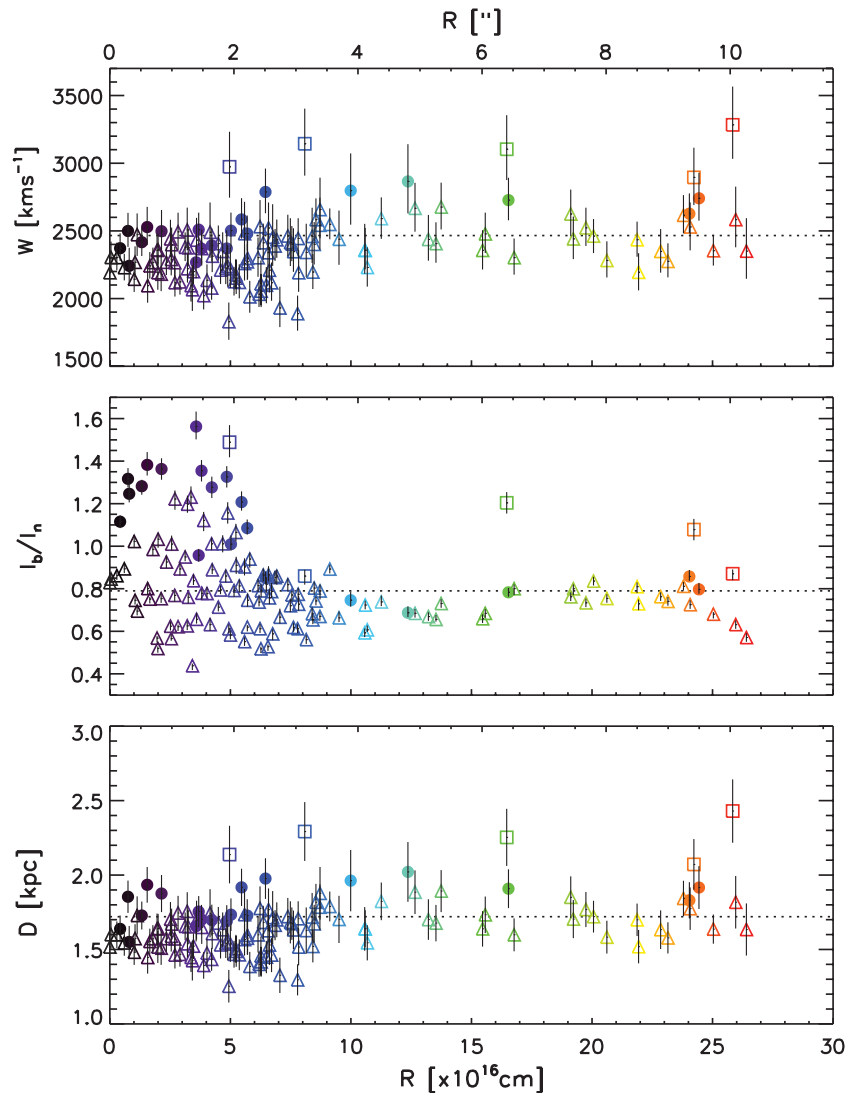
right) The reduced data cube collapsed in wavelength around the H $\alpha$  line, recovering the shock front. The crosses with four different colors indicate the pixels for which the spectra have been combined to produce the spectra at the bottom. (Bottom) Dashed black lines show the best-fit double-Gaussian with given parameters.  $\Delta V$  and  $W$  were measured in  $\text{km s}^{-1}$ . The reduced  $\chi^2$  values above unity, along with the differences between the observed spectra and their best fits (shown below the spectra with an offset of  $-0.3$  for clarity), indicate that non-Gaussianity is present. Most of the reduced  $\chi^2$  values above unity come from the mismatching near the line core (insets) from  $-1000$  to  $1000 \text{ km s}^{-1}$ . On the horizontal axis is shown only the fitted region of the spectra, whereas the y axis shows the flux in units of  $10^{-16} \text{ erg s}^{-1} \text{ cm}^{-2} \text{ \AA}^{-1}$ , rescaled with respect to the blue graph by factors 2 and 0.5 for the yellow and red graphs, respectively.



**Fig. 2. Two-dimensional spatial-spectral maps of various properties associated with the shock front in the remnant of SN 1006. (Bottom right)** The spatial Voronoi binning to reach a minimum  $S/N \approx 40$ . By fitting a double Gaussian to the resulting combined spectrum in each bin, we obtained  $W$  (**top left**),  $I_b/I_n$  (**top middle**), and  $\Delta V$  converted into  $\theta$ . By using shock models, we converted  $W$  and  $I_b/I_n$  values into  $v_s$  (**bottom left**) and  $\beta$  (**bottom middle**).



**Fig. 3. All measured  $W$ ,  $I_b/I_n$ , and heliocentric distances ( $D$ ) from combining the proper motion measurement with the shock velocities. Data (with error bars) are ordered in increasing distance from the inner rim ( $R$ ) (black and purple) to the outer rim (orange and red), shown in arc sec at the top and in units of  $10^{16}$  cm at the bottom horizontal axis. The inner rim coincides with the inner edge of the blue rim in Fig. 1, top right. The dashed horizontal lines indicate the measured  $W = 2465.76 \text{ km s}^{-1}$  and  $I_b/I_n = 0.79$  values from collapsing all spectra of the pixels on the shock front, and from there the inferred  $D = 1.72 \text{ kpc}$ . Data points marked with a solid circle are those for which a valid model solution is found. The triangles indicate that, because of low  $I_b/I_n$  values, the models hit the lower boundary limit in  $\beta$  of 0.01, whereas squares indicate that high  $I_b/I_n$  values caused the models to hit the upper boundary limit in  $\beta$  of 1.0.**



lack of nonthermal x-ray and TeV  $\gamma$ -ray emission in the northwestern rim of the remnant indicates that, if there is a cosmic ray precursor, the injection of electrons is little and the particle energies are low ( $<1$  MeV), but cosmic ray acceleration can still happen.

Our pilot project demonstrates the feasibility of using integral-field spectroscopy to observe and study the microphysics of high-velocity shocks around supernova remnants. The resulting high spatial resolution mapping of the Balmer-dominated shocks in the northwestern rim of SN 1006 suggests the presence of suprathermal protons of energies 10 to 100 keV, which can seed high-energy cosmic rays.

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## Supplementary Materials

[www.sciencemag.org/cgi/content/full/science.1228297/DC1](http://www.sciencemag.org/cgi/content/full/science.1228297/DC1)  
Materials and Methods  
Supplementary Text  
Figs. S1 to S3  
Table S1  
References (30–33)

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# A Tissue-Like Printed Material

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Living cells communicate and cooperate to produce the emergent properties of tissues. Synthetic mimics of cells, such as liposomes, are typically incapable of cooperation and therefore cannot readily display sophisticated collective behavior. We printed tens of thousands of picoliter aqueous droplets that become joined by single lipid bilayers to form a cohesive material with cooperating compartments. Three-dimensional structures can be built with heterologous droplets in software-defined arrangements. The droplet networks can be functionalized with membrane proteins; for example, to allow rapid electrical communication along a specific path. The networks can also be programmed by osmolarity gradients to fold into otherwise unattainable designed structures. Printed droplet networks might be interfaced with tissues, used as tissue engineering substrates, or developed as mimics of living tissue.

Cells in living tissues communicate with each other in a controlled manner. As a consequence, tissues display the emergent properties that distinguish them from collections of independently functioning cells. The reproduction of similarly sophisticated behavior in a synthetic system would be of scientific and technological value but is not feasible with existing artificial membrane platforms. Of the various classes of structures containing artificial bilayers, liposomes resemble cells most closely, but the controlled insertion of specialized membrane proteins such as gap junctions (1) would be required to join liposomes and form a cohesive, cooperative system.

Lipid-coated aqueous droplets in oil adhere at their interfaces to form stable bilayers (2–5), which can be functionalized with membrane proteins.

Small two-dimensional (2D) networks of droplets connected in this way have been shown to act cooperatively as light sensors (5), batteries (5), or simple electrical circuits (6). Further, the droplets can release their contents to bulk aqueous solution after a change in pH or temperature (7–9). Droplet networks are therefore a promising platform for the construction of complex functional devices. However, functional networks have been limited to small groups of droplets assembled by manual (5–7) or mechanical (10) manipulation, microfluidic means (3, 11, 12), or external fields (13–15). Larger assemblies have been constructed by packing droplets into microfluidic containers (16, 17), but their complexity is limited by the uncontrolled filling process.

We automatically printed tens of thousands of heterologous picoliter droplets in software-defined, 3D millimeter-scale geometries. The resulting macroscopic material is cohesive and self-supporting and consists of distinct aqueous compartments partitioned by single lipid bilayers. Printing can take place in bulk oil or within oil

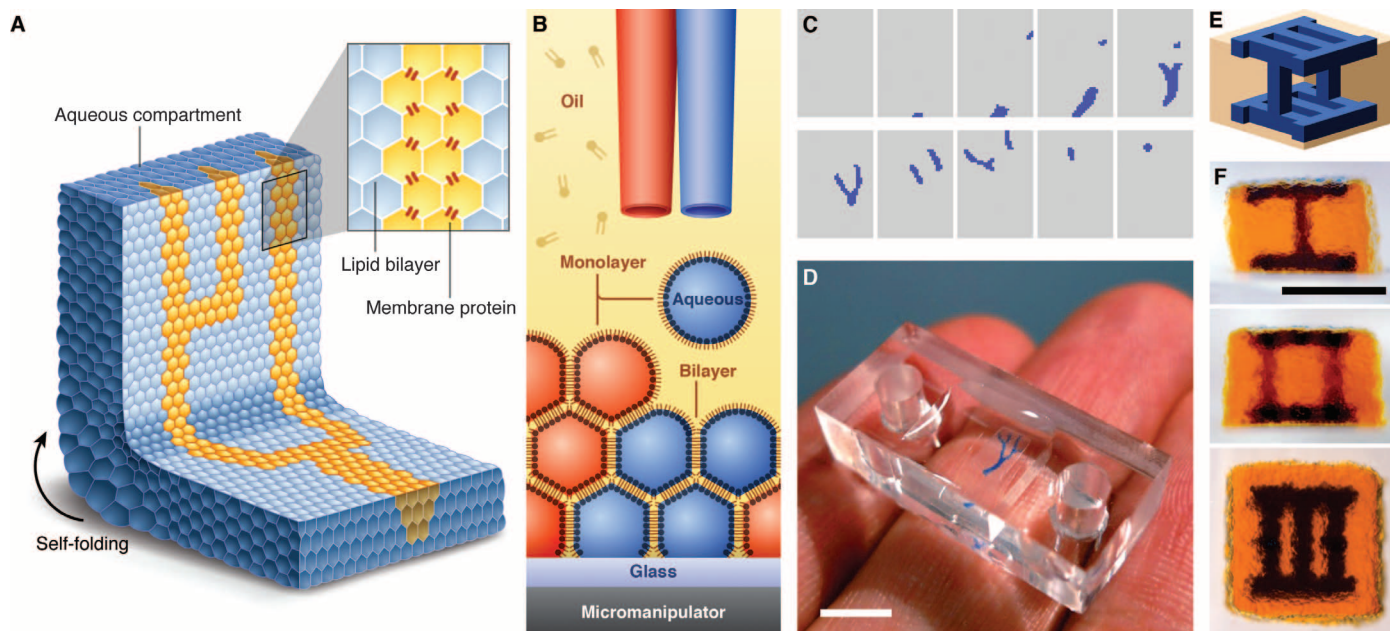
drops that reside in aqueous solution. The bilayers can be functionalized with membrane proteins to allow electrical communication along a specific route. Printed droplet networks can also be programmed by osmolarity gradients to fold after printing into various designed geometries not accessible by direct printing. Three characteristics distinguish printed networks from other shape-changing materials such as the bimetallic strip or hydrogels patterned to undergo non-uniform volume changes under external stimuli (18, 19): droplet networks are readily printed, they consist of compartments that can communicate through membrane proteins, and their folding is driven by internal differences in osmolarity. The latter characteristics make the folding behavior of droplet networks closely analogous to the nastic movements exhibited by certain plants (20, 21). Printed picoliter droplet networks constitute a defined synthetic platform for sophisticated collective behaviors (Fig. 1A) and might be developed for medical applications (22).

Our strategy was to eject aqueous droplets (fig. S1 and supplementary text S1) within a bath of lipid-containing oil that was mounted on a motorized micromanipulator, so that a droplet network was built up in horizontal layers (Fig. 1B). The construction of droplet networks raises challenges that preclude the use of a commercially available printer (supplementary text S2), which we addressed with a specially designed system (23) (figs. S2 to S8 and table S1). A network is defined in horizontal cross sections one droplet thick (Fig. 1C), and a custom computer program (23) accordingly synchronizes the motion of the oil bath with the ejection of droplets from two droplet generators to construct the desired network (Fig. 1D). We have printed precisely defined networks consisting of up to

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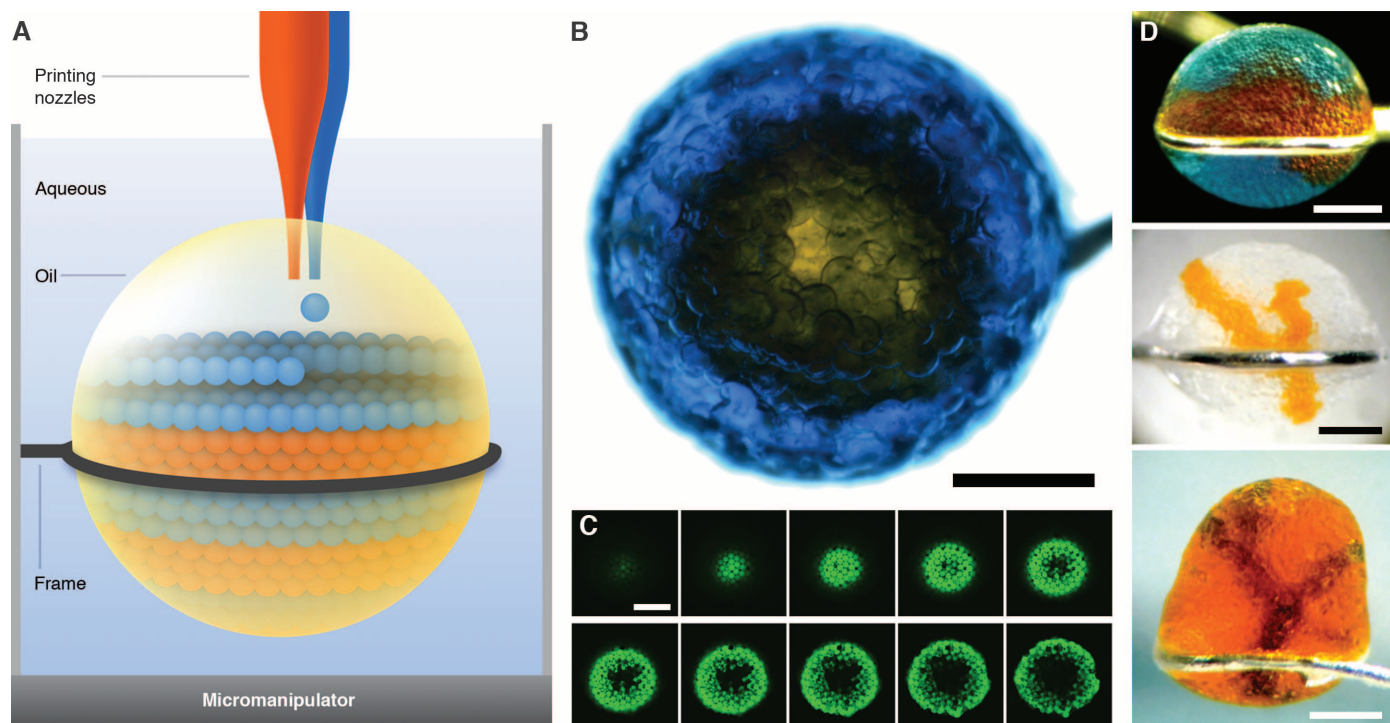
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**Fig. 1. Printed droplet networks.** (A) Illustration of a printed droplet network. (B) Schematic of the printing process. Two droplet generators eject droplets, typically ~65 pL, of different aqueous solutions into a solution of lipids in oil. The oil bath is mounted on a motorized micromanipulator. The droplets acquire a lipid monolayer and form bilayers with droplets in the growing network. (C) Horizontal cross-sections of a design for a 3D droplet

network with a branching structure (blue) embedded in a cuboid (gray). The design comprises 20 layers of  $50 \times 35$  droplets each; only alternate layers are shown. (D) Network printed according to the design in (C). Scale bar, 5 mm. (E) Schematic of a 3D design that consists of 28 layers of  $24 \times 24$  droplets each. (F) Three orthogonal views of a single network printed according to the design in (E). Scale bar, 1 mm.



**Fig. 2. Droplet networks printed in bulk aqueous solution.** (A) Schematic of printing in aqueous solution. Aqueous droplets are ejected into a drop of oil suspended in bulk aqueous solution. Excess oil can be removed after printing by suction through a printing nozzle. (B) Micrograph of a network printed in aqueous solution, viewed from above. A core of orange droplets is surrounded by a shell of blue droplets, which contain the

fluorescent dye pyranine. Scale bar, 400  $\mu\text{m}$ . (C) Horizontal sections of the network in (B) obtained by confocal microscopy, showing the fluorescent shell of droplets around the nonfluorescent core. The sections span approximately the bottom 150  $\mu\text{m}$  of the network. Scale bar, 400  $\mu\text{m}$ . (D) Micrographs of three other networks printed in bulk aqueous solution. Scale bars, 400  $\mu\text{m}$ .

~35,000 heterologous droplets ejected at a rate of  $\sim 1 \text{ s}^{-1}$  (Fig. 1, C to F).

Printed droplet networks are self-supporting (Fig. 1, D and F), and a thermodynamic analysis of the system indicates that stable networks can be printed with at least several thousand layers (23). The lattice of lipid bilayers also allows droplet networks to retain their shape under gentle perturbation; each bilayer lends an effective spring constant of  $\sim 4 \text{ mN m}^{-1}$  to connected droplets, with a tensile strength of  $\sim 25 \text{ Pa}$  (23) (figs. S9 and S10). We estimate that Young's modulus of printed networks is on the order of  $\sim 100$  to  $200 \text{ Pa}$  (23) (fig. S11), which is comparable to the elastic moduli of brain, fat, and other soft tissues (24).

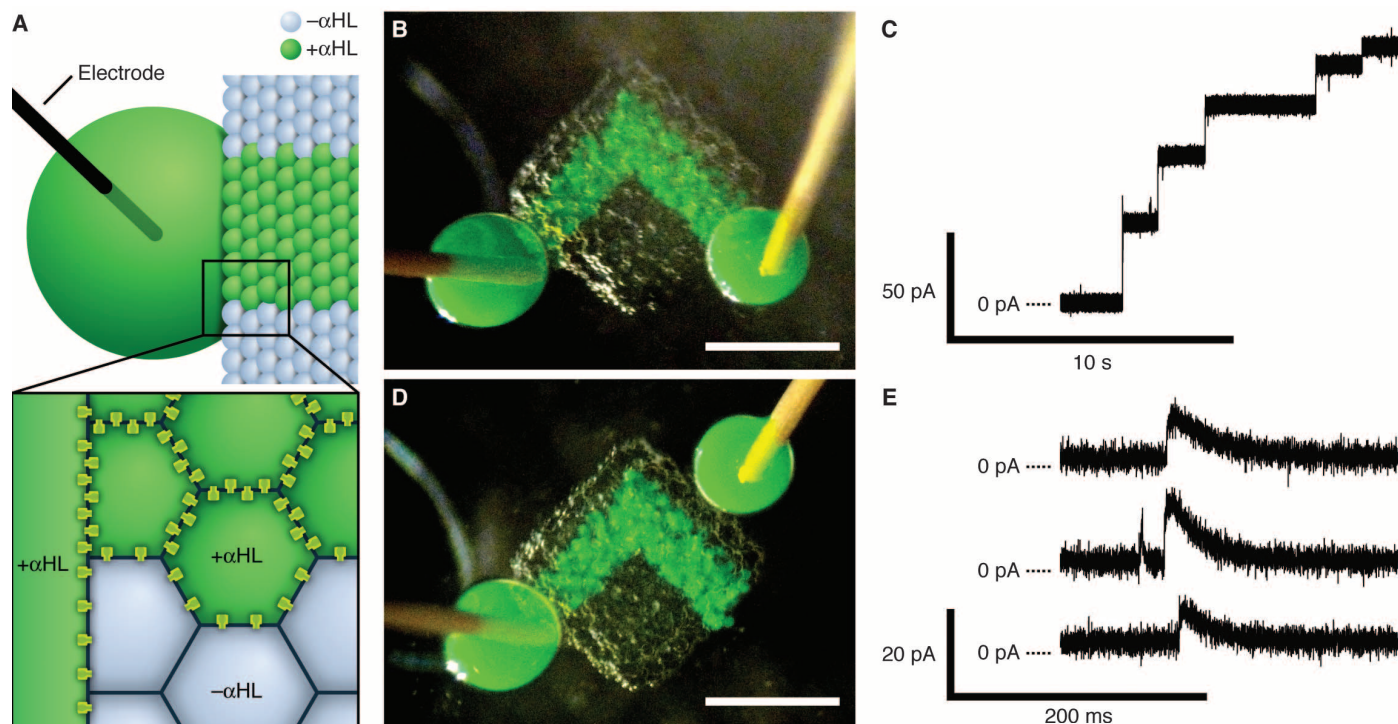
We have shown (7) that droplet networks can be stabilized in bulk aqueous solution by encapsulation within small drops of oil, for prospective applications in synthetic biology and medicine. Whereas those networks were created manually and therefore were limited in complexity, in the present experiments, we demonstrated the printing of encapsulated networks consisting of thousands of droplets in designed 3D patterns. This was achieved by printing inside an oil drop suspended in aqueous solution (23) (Fig. 2A and supplementary text S3). Once printing is complete, excess oil can be removed by suction through one of the printing nozzles. Encap-

sulated printed networks (Fig. 2, B to D) were stable for at least several weeks and will therefore serve to expand the functions previously demonstrated with simple encapsulated networks, including communication with the aqueous surroundings through membrane pores, and pH- or temperature-triggered release of contents (7).

To establish whether membrane proteins could be included in specific bilayers, we printed a network in which only the droplets along a defined pathway contained staphylococcal  $\alpha$ -hemolysin ( $\alpha$ HL), in order to create an ionically conductive route across an otherwise insulating network (Fig. 3, A and B). To probe the network electrically, a drop of buffer of  $\sim 500 \text{ }\mu\text{m}$  in diameter containing  $\alpha$ HL pores was manually pipetted onto each of two Ag/AgCl electrodes with agarose-coated tips. The drops were then brought into contact with different parts of the network, so that they formed bilayers with the droplets on the network surface (Fig. 3A). When the two large drops were placed on either end of the  $\alpha$ HL-containing pathway (Fig. 3B), we measured a stepwise increase in ionic current under an applied potential (Fig. 3C). After one of the drops was separated and brought back into contact with the network away from the  $\alpha$ HL-containing pathway (Fig. 3D), only transient currents were observed (Fig. 3E). When this drop was separated from the network again

and replaced in its original position, a stepwise increase in current was again observed (fig. S12). Droplet networks in which no droplets contained  $\alpha$ HL showed negligible current flow, whereas the current measured across droplet networks in which every droplet contained  $\alpha$ HL was similar to that shown in Fig. 3C (fig. S12).

To interpret these results, we performed computational simulations of the electrical behavior of printed droplet networks (23) (fig. S13). The electrical model was consistent with the measured currents exemplified in Fig. 3, C and E, provided that most of the bilayers along the  $\alpha$ HL-containing pathway contained several pores and the other bilayers in the network contained none (supplementary text S4 and fig. S14), so that the pathway presented an already established conductive route through the otherwise insulating network. The stepwise increase in current in Fig. 3C was most likely caused by pore insertions into the bilayers between the large electrode drops and the pathway droplets, rather than into bilayers within the printed network (supplementary text S4). The current spikes in Fig. 3E correspond to pore insertions into the bilayers between the electrode drop newly placed away from the pathway and insulating droplets in the network, which transmit transient capacitive currents but do not permit a steady resistive current (supplementary text S4). Based on these findings,



**Fig. 3. Electrically conductive pathway.** (A) Schematic of part of a network printed with an ionically conductive pathway. Only the green droplets and the large drop contain  $\alpha$ HL pores. The large drop is impaled with an Ag/AgCl electrode. The magnified section illustrates the  $\alpha$ HL pores in the bilayers around the  $\alpha$ HL-containing droplets. (B) Photograph of a printed network with electrode-impaired drops placed on either end of the conductive pathway. The green droplets contain  $\alpha$ HL, whereas the other

droplets contain no protein. Scale bar,  $500 \text{ }\mu\text{m}$ . (C) Stepwise increase in the ionic current as measured in the configuration in (B), at  $50 \text{ mV}$  in  $1 \text{ M KCl}$  at  $\text{pH } 8.0$ . (D) Photograph of the network in (B), after separating one of the large drops and rejoining it onto the network away from the conductive pathway. Scale bar,  $500 \text{ }\mu\text{m}$ . (E) Selected portions of a single recording as measured in the configuration in (D) at  $50 \text{ mV}$ , showing transient increases in ionic current.



we maintain that droplet networks can be printed with protein pores in specific bilayers. The tolerance of printed networks to slight variations in structure and in the number of proteins that insert in each bilayer may be compared to the robustness of living tissues to minor flaws. The printed network presented here is functionally analogous to a nerve axon in enabling rapid, long-distance electrical communication along a defined path, but it does not mimic an axon's mechanism of signal propagation.

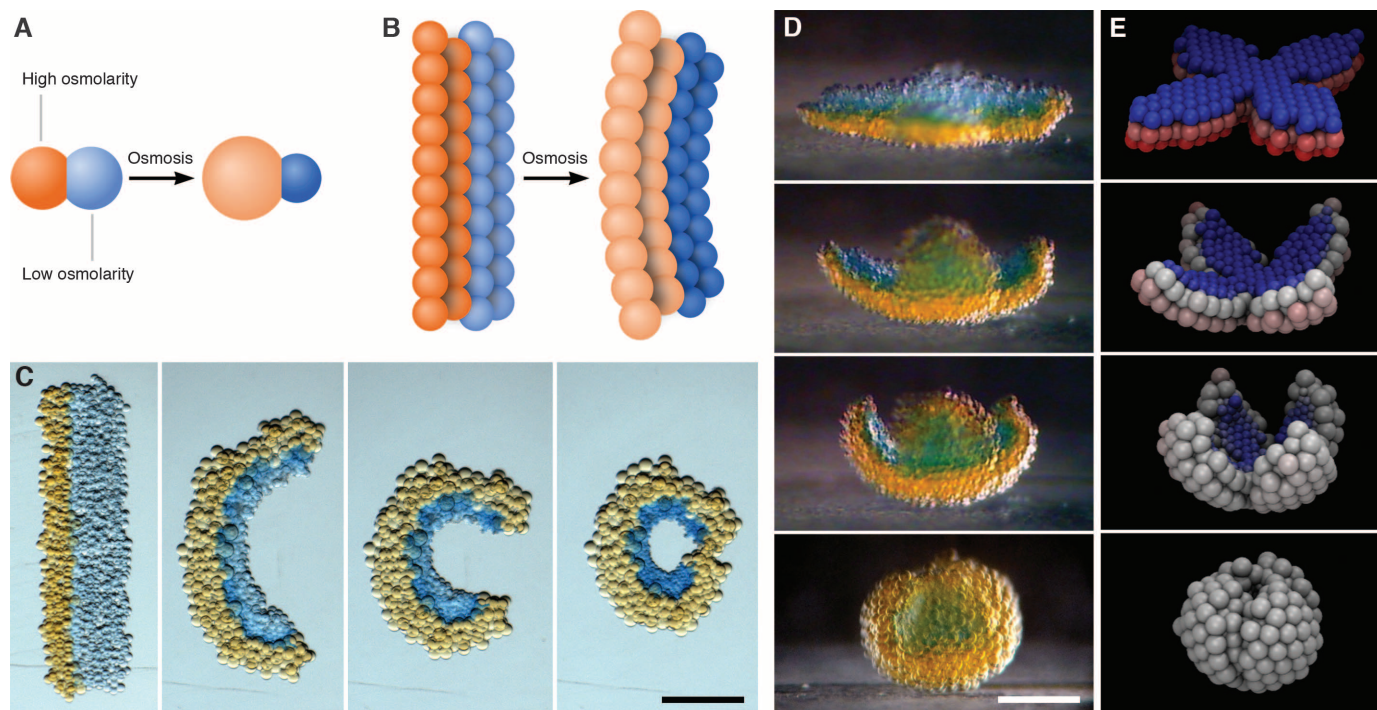
Finally, we explored a means for droplets in a printed network to produce a designed macroscopic change in the network geometry through cooperative action. Water permeates readily through droplet interface bilayers even in the absence of protein channels or pores, with a permeability coefficient of  $27 \pm 5 \mu\text{m s}^{-1}$  (mean  $\pm$  SD,  $n = 3$  pairs of droplets joined by bilayers) under the conditions of this study (fig. S15), consistent with other permeability measurements of droplet interface bilayers (25–27) and other lipid bilayer systems (28). Consequently, two droplets of higher and lower osmolarity joined by an interface bilayer will respectively swell and shrink until their osmolarities are equal (Fig. 4A). By extension, water transfer within a network composed of droplets of different osmolarities will cause spontaneous deformation of the network as long as adhesion between droplets is maintained (Fig. 4B).

We found several prerequisites for droplet networks to fold in a predictable way (see also supplementary text S5). First, to prevent droplets from being printed onto incorrect positions in the network, the network must fold slowly as compared to the printing time. Second, the swelling and shrinking of the two droplet types induces a stress between regions of connected droplets that can cause the network to buckle in an uncontrolled manner. This is analogous to the buckling instability in tissues that grow at inhomogeneous rates, such as certain leaves (29) and flower petals (30). In certain cases, the stress may instead cause connected droplets to separate and thereby prevent the network from folding further around the fracture zone (fig. S16). These various problems can be solved through judicious choices of printing rate, salt concentrations, and droplet size, and adjustments to the network geometry (supplementary text S5). The final geometry of the network is then determined in a well-defined way by its initial geometry, the distribution of the two types of droplets, and the ratio of their osmolarities. Using a simple computational model that allows us to predict the folding behavior of a given droplet network (23) (table S2), we designed droplet networks that folded successfully.

In one experiment, we printed a network that comprised two strips of droplets of different salt concentrations, connected along their lengths

(Fig. 4C). The network folded spontaneously in the horizontal plane over  $\sim 3$  hours, until droplets at opposing ends of the network formed new bilayers in a closed ring. We also programmed a network to fold spontaneously out of the horizontal plane to attain a geometry that would be difficult to print directly. We printed a flower-shaped network with four petals, in which the lower layers had a higher osmolarity than the upper layers. The permeation of water from the upper into the lower layers induced a curvature that raised the petals and folded them inward (Fig. 4D and movie S1). The folded network was self-supporting and approximated the geometry of a hollow sphere, with the originally upper layer contained within a shell formed by the originally lower layer (Fig. 4E and fig. S17). The evolution of the geometry of the network is in good agreement with that of a simulated folding network with similar initial conditions (Fig. 4E and movie S2). We estimate the energy available from the osmosis-driven flow of water to be on the order of  $1 \mu\text{J}$  for this network (23), which is comparable to the total energy of bilayer formation in the network and is several orders of magnitude greater than the energy required to lift the droplets against gravity (23).

We have devised a means of printing, in designed 3D geometries, a soft material composed of aqueous microcompartments bound together by lipid bilayers. The bilayers allow the



**Fig. 4. Self-folding droplet networks.** (A) Schematic of two droplets of different osmolarities joined by a lipid bilayer. The flow of water through the bilayer causes the droplets to swell or shrink. (B) Schematic of a droplet network that comprises two strips of droplets of different osmolarities. The transfer of water between the droplets induces an overall deformation of the network. (C) Photographs of a rectangular network folding into a circle over  $\sim 3$  hours. The orange and blue droplets initially contained 250 mM KCl

and 16 mM KCl, respectively. Scale bar, 250  $\mu\text{m}$ . (D) Photographs of a flower-shaped network folding spontaneously into a hollow sphere. The orange and blue droplets initially contained 80 mM KCl and 8 mM KCl, respectively. The photographs cover a period of 8 hours. Scale bar, 200  $\mu\text{m}$ . (E) Frames from a folding simulation of a network with a similar initial geometry to the network in (D). Blue and red represent the lowest and highest initial osmolarities, respectively, and white indicates the average of the two.

compartments to interact directly through membrane proteins or osmotic flows of water and thereby enable the engineering of collective properties such as long-range electrical communication or macroscopic deformation. We used the well-characterized  $\alpha$ HL pore and simple salt solutions to achieve cooperative action between droplets in a printed droplet network. Additional membrane proteins and their engineered forms (5, 6) should allow printed networks to transduce a wider range of signals, and stimulus-responsive osmolytes might offer greater control of folding. An interesting challenge is the integration of droplet networks with living organisms. The outer surface of a printed network might be engineered to interact in a designed way with a physiological environment; for example, to deliver drugs upon a specific signal (7–9). More sophisticated networks might be interfaced with failing tissues to support their functions. Alternatively, cells could be included inside the droplets during printing for various applications, such as to immobilize cells within a defined 3D dimensional scaffold for tissue engineering (22).

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#### Supplementary Materials

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## Broadband 2D Electronic Spectroscopy Reveals a Carotenoid Dark State in Purple Bacteria

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Although the energy transfer processes in natural light-harvesting systems have been intensively studied for the past 60 years, certain details of the underlying mechanisms remain controversial. We performed broadband two-dimensional (2D) electronic spectroscopy measurements on light-harvesting proteins from purple bacteria and isolated carotenoids in order to characterize in more detail the excited-state manifold of carotenoids, which channel energy to bacteriochlorophyll molecules. The data revealed a well-resolved signal consistent with a previously postulated carotenoid dark state, the presence of which was confirmed by global kinetic analysis. The results point to this state's role in mediating energy flow from carotenoid to bacteriochlorophyll.

Carotenoids (Cars) play an important role in the photoreactions of photosynthesis (1). Being accessory pigments, they absorb light in the spectral region not accessible to chlorophylls (Chls) and, by subsequent energy transfer to Chls, they increase the spectral cross-section for photosynthetic activity. Moreover, in

some organisms, most of the light that drives photosynthesis is absorbed by Cars (2). However, in spite of many years of studies, the exact mechanism of energy transfer from Cars to Chls remains controversial (3, 4), owing to the complex electronic structure of Cars.

Traditionally, Car photophysics is described by a three-level model, in which the ground state-to- $S_1$  ( $2A_g^-$ ) state transition is symmetry-forbidden and the light is absorbed by the  $S_2$  ( $1B_u^+$ ) state. After excitation, the  $S_2$  state rapidly decays in a few hundred femtoseconds to the “dark”  $S_1$  state, which then decays back to the ground state ( $S_0$

or  $1A_g^-$ ) on a picosecond time scale. However, the picture becomes more complicated for Cars with nine or more conjugated double bonds, for which Tavan and Schulten predicted the appearance of other dark states between the  $S_2$  and  $S_1$  states (5), in particular, the  $1B_u^-$  state. In 2002 Cerullo *et al.* (6), using sub-10-fs pulses to excite isolated Cars, reported the presence of a kinetic intermediate phase (called  $S_x$ ) that was populated by decay of the  $S_2$  state and then decayed into the  $S_1$  state. However, the interpretation of this phase as the dark electronic state has been controversially discussed in later studies (7, 8). Moreover, it remained unclear whether such an intermediate electronic state is actually involved in Car-to-Chl energy transfer.

In this work we investigated how the complex manifold of Car electronic states in the peripheral light-harvesting complex LH2 from the purple bacteria *Rhodospseudomonas acidophila* strain 10050 and *Rhodobacter sphaeroides* strain 2.4.1 couple to the  $Q_x$  state of bacteriochlorophyll (BChl). By comparing the 2D spectra of LH2 complexes with spectra of isolated Cars, we deduce the presence of a Car dark state in the vicinity of the  $Q_x$  state. A global kinetic analysis suggests several energy transfer pathways associated with this dark state. The results demand reexamination of the intermolecular energy transfer mechanisms in light-harvesting proteins.

The molecular structure of the LH2 complex from *Rps. acidophila* and corresponding ladder of

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electronic energy levels are depicted in Fig. 1. For the LH2 complexes of purple bacteria, light absorption mainly populates the  $Q_x$  and  $Q_y$  excited states of BChl-a (BChl) and the high-lying  $S_2$  excited state of the Car. In order to study the energy transfer between the high-lying states of isolated LH2 complexes, we applied broadband 2D electronic spectroscopy. The excitation conditions were specifically designed to probe simultaneously the Car  $S_2$  and BChl  $Q_x$  excited states. After excitation, the Car  $S_2$  state either relaxes to the lowest-lying Car excited state, the dark  $S_1$

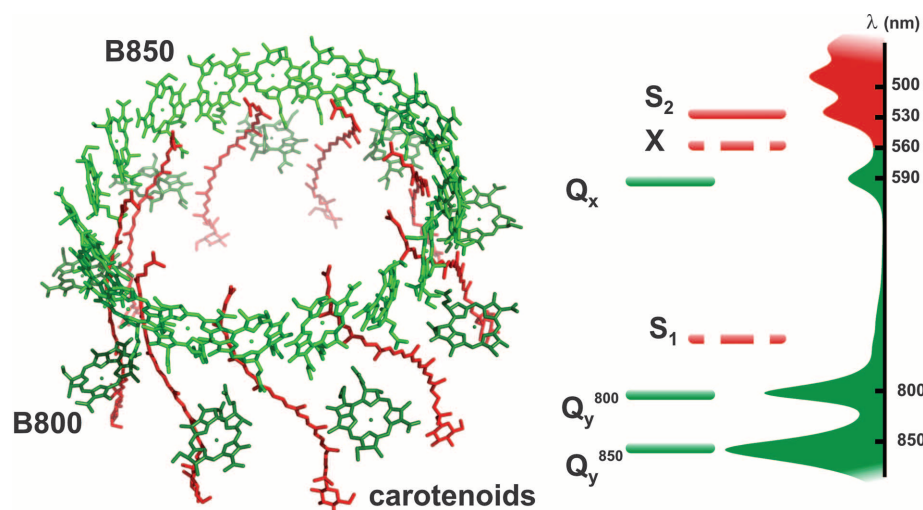
state, or else transfers energy to the BChl  $Q_x$  state. The efficiency of the  $S_2 \rightarrow Q_x$  energy transfer is experimentally estimated to be between 40 and 60% (9–12). An additional channel of energy flow from the Car  $S_1$  dark state to BChl  $Q_y$  accounts for 4 to 10% (11, 13). Even higher values of the overall Car-to-BChl energy transfer efficiency are achieved in *Rba. sphaeroides* (~90%), where a substantial fraction of the energy is transferred from the  $S_1$  state (14, 15). Theoretical studies, however, predicted only 20% efficiency for the overall Car-to-BChl energy transfer (16). The

disagreement between theoretical predictions and experimental observations is ascribed to the small spectral overlap of  $S_2$  emission and  $Q_x$  absorption bands, which results from the large energy gap between these transitions.

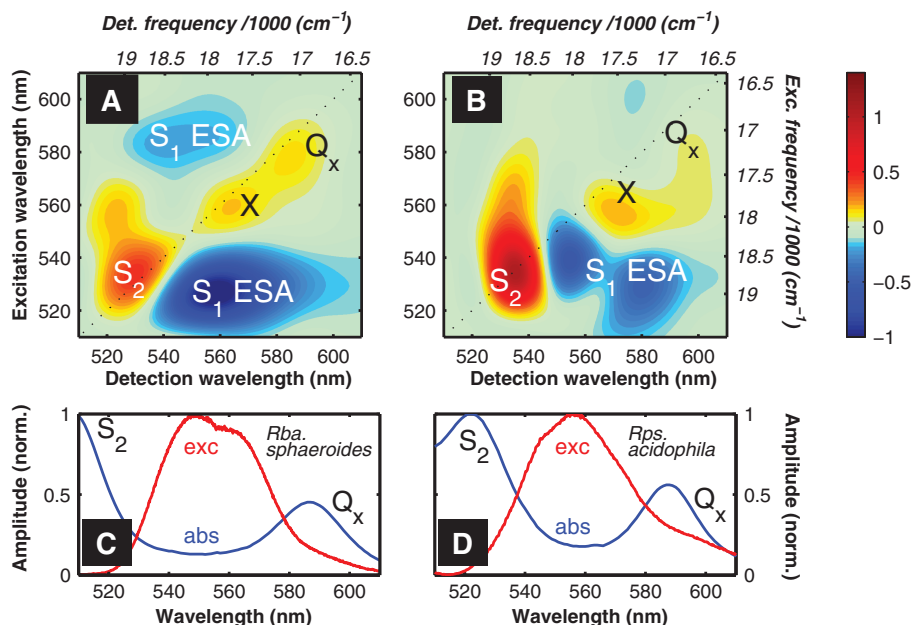
It is a challenge to elucidate questions concerning the nature and significance of dark electronic states using normal femtosecond pump-probe experiments, because their signals are overwhelmed by contributions from the strongly allowed transitions: ground-state bleach (GSB) and stimulated emission (SE) from the Car  $S_2$  and BChl  $Q_x$  states, as well as excited-state absorption (ESA) from the Car  $S_1$  state. We took advantage of the combined time and frequency resolution attainable in 2D electronic spectroscopy (17, 18) that enables sensitive detection of coupling among states via cross-peaks. Therefore, we could detect not only the spectral signatures and kinetics of various electronic states, but also their interaction pathways.

In our experiment, excitation pulses of broad bandwidth (full width at half maximum 50 to 60 THz, 13 to 15 fs) were applied in the 500- to 650-nm spectral range. The LH2 complexes of *Rps. acidophila* and *Rba. sphaeroides* were selected because of the relatively small energy gap between the  $S_2$  state and  $Q_x$  state (65 to 80 nm), which allowed our excitation pulses to overlap the transitions to these two states simultaneously. The spectral width and central wavelength of the pulses were optimized carefully to cover both the Car  $S_2$  and BChl  $Q_x$  transitions in the LH2 absorption spectrum. The 2D spectra were obtained by measuring the spectral response of the system at different delays between the first two pulses ( $t_1$ , the so-called coherence time). The resulting 2D data were Fourier-transformed with respect to  $t_1$ , yielding the excitation frequency scale. This procedure was repeated for different delays between second and third pulses ( $t_2$ , the so-called population time) in order to obtain the temporal evolution of the spectral response. Figure 2, A and B, shows 2D spectra of *Rba. sphaeroides* and *Rps. acidophila* taken at  $t_2 = 215$  and 158 fs, respectively.

The excitation pulse spectra are shown in Fig. 2, C and D, together with the absorption spectra of the corresponding LH2 complexes. Several well-resolved features are evident in the 2D spectra. Three positive peaks on the diagonal are due to the GSB signal. In accordance with the stationary absorption spectra, the two peaks at 530 and 580 nm are assigned to excitation to the Car  $S_2$  and BChl  $Q_x$  states, respectively. Owing to stronger overlap of the excitation pulse with the  $S_2$  absorption band in *Rps. acidophila*, the intensity of the corresponding diagonal peak is much more pronounced than in *Rba. sphaeroides*. The off-diagonal signal with negative amplitude located below the diagonal is due to the ESA signal from the Car  $S_1$  state. This feature of Cars is well known from transient absorption studies (12) and reflects the  $S_2 \rightarrow S_1$  energy transfer pathway. The negative off-diagonal signal located



**Fig. 1. Molecular structure, absorption spectrum, and scheme of corresponding electronic levels of LH2 complexes of *Rps. acidophila*.** BChl-a (B800 and B850) are shown in green, Cars in red. Dashed lines at right correspond to states inaccessible by direct optical excitation from the ground state.



**Fig. 2. Absorptive 2D spectra of (A) *Rba. sphaeroides* taken at  $t_2 = 215$  fs and (B) *Rps. acidophila* taken at  $t_2 = 158$  fs.** Spectra of *Rba. sphaeroides* were measured over a range from 0 to 400, with 5-fs steps; spectra of *Rps. acidophila* were measured over a range from 0 to 200 fs, with 1-fs steps. Absorption spectra (blue line) and excitation pulse spectra (red line) of (C) *Rba. sphaeroides* and (D) *Rps. acidophila* are shown.

above the diagonal in the *Rba. sphaeroides* spectrum might be due either to ESA from the Car  $S_1$  state, populated from the BChl  $Q_x$  state as was proposed by Kosumi *et al.* (19), or due to ESA from BChl  $Q_y$ , populated by internal conversion from the  $Q_x$  state (20). The observed signal could also be a product of both of these two signals. In order to distinguish between these two contributions, a kinetic analysis has to be applied. In 2D spectra recorded for *Rps. acidophila*, only one ESA signal is observable. It is located below the diagonal and is associated with excitation of the Car  $S_2$  state. The ESA signal above the diagonal is generally a weaker feature, and it is obscured in raw data owing to overlap with positive  $Q_x/S_2$  and  $Q_x/X$  cross-peaks, which have higher amplitude in *Rps. acidophila* than in *Rba. sphaeroides*. However, as will be shown below, the extraction of the oscillatory component and application of the kinetic analysis uncover a weak  $S_1$  ESA signal due to  $Q_x \rightarrow S_1$  energy transfer [Fig. 4D and (21)]. The double-peak shape of the ESA in Fig. 2B has the same origin, because it overlaps with the positive  $S_2/X$  cross-peak.

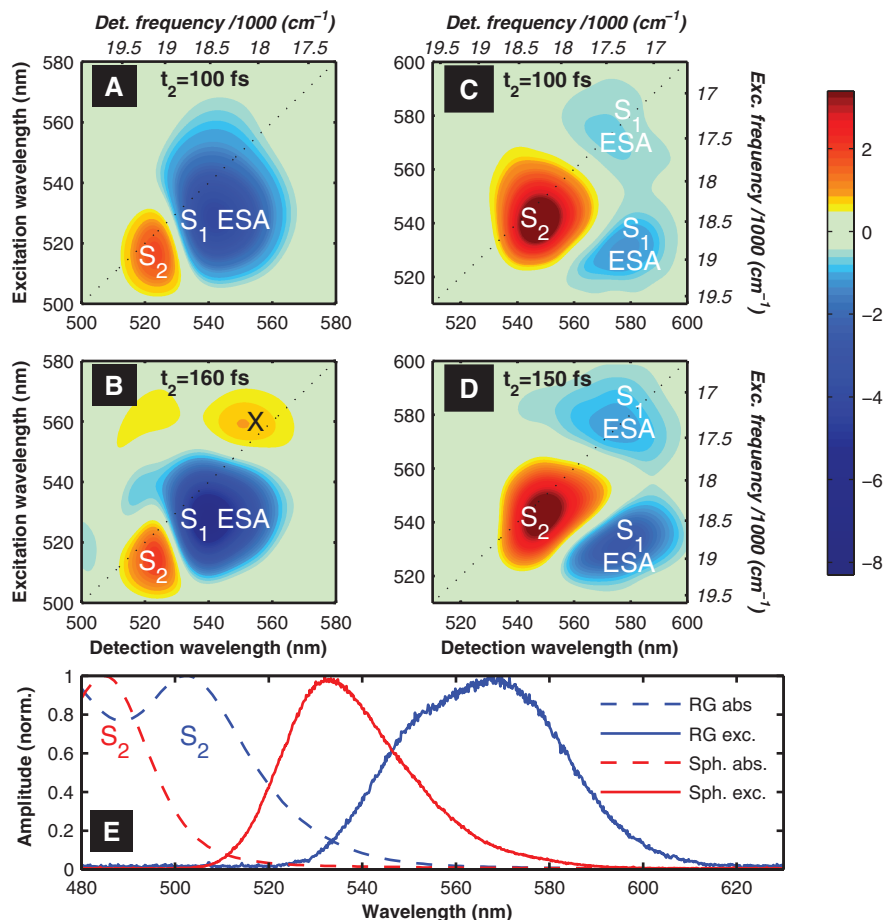
Positive cross-peaks in 2D spectroscopy are caused either by coupling of the corresponding states (i.e., via a common ground state) or by energy transfer between the states. Cross-peaks between  $S_2$ ,  $Q_x$ , and X states can be observed at different  $t_2$  delay times and are shown in figs. S5 to S8 as well as in movies S1 and S2 (these auxiliary measurements were performed over a 0- to 400-fs time range, with 5-fs time steps). These data illustrate two points. One is that the 2D spectra are complicated because of the oscillations and the number of transitions that contribute to the spectroscopic map. The second point is that, despite the complexity, there are a sufficient number of clearly identified transitions to allow fitting of these data using a global target analysis. First we point out some qualitative clues to the origin of the X state.

The diagonal signal marked X as well as the cross-peaks at  $\sim 560$  nm excitation and detection have not been observed previously (e.g., in transient absorption studies). In order to elucidate the origin of the corresponding state, namely to determine whether it is due to Car or BChl and whether it is a vibrational sublevel or an electronic state, we performed Fourier analysis of the data along the  $t_2$  time variable. The time traces taken at points along the diagonal of the 2D spectrum of *Rps. acidophila* at the peak positions (at 530-, 560-, and 585-nm excitation/detection wavelengths, see fig. S9A) reveal pronounced oscillations. The oscillations at the 530- and 560-nm peaks show clear beating, indicating the presence of more than one frequency. The Fourier spectra of these two traces are almost identical and reveal three high-frequency modes (fig. S9B). These three modes of 990, 1195, and  $1590\text{ cm}^{-1}$  ( $\text{CH}_3$  rocking mode, C-C stretching mode, and C=C stretching mode, respectively) are known to be signature vibrational frequencies of the Car ground state (10, 22).

The Fourier spectrum of the trace sampled at the 585 nm peak ( $Q_x$  bleach) strongly differs from the other two. It has only one high-frequency mode,  $1590\text{ cm}^{-1}$ . The presence of that Car mode at that coordinate is due to overlapping Car signal. Indeed, as follows from the 2D maps of Fourier spectra of *Rps. acidophila* shown in fig. S10, the diagonal peak at the 560-nm coordinate has noticeable elongation to longer wavelengths, with significant amplitude at 585 nm. The BChl is not known to have pronounced ground state vibrational modes in the high-frequency region. Only a weak  $1340\text{ cm}^{-1}$  mode can be assigned to the BChl ground state, based on comparison of the resonance Raman spectra of isolated BChl molecules and LH2 complexes (23). A small peak in fig. S9B observed at  $1370\text{ cm}^{-1}$  frequency is most probably due to that BChl mode; however, due to the much larger contribution of the Car vibrational modes, this peak cannot be identified with high certainty. Thus, the comparison of the Fourier spectra taken at the diagonal indicates that the X peak is associated with a Car state. Similar features are observed in the case of *Rba. sphaeroides* (fig. S11).

In order to ensure that the X state is indeed a Car feature, we performed 2D spectroscopy measurements on the isolated Cars rhodopin glucoside (from *Rps. acidophila*) and spheroidene (from *Rba. sphaeroides*), both in acetone solution. These Cars have different conjugation length, which causes differences in their spectroscopic features. Figure 3 shows 2D spectra of the two Cars taken at  $t_2 = 100$  fs and 150 to 160 fs. The Cars were excited at the very red edge of the absorption spectrum in order to minimize the contribution of the  $S_2$  state GSB in the 2D spectrum (Fig. 3E). For such excitation conditions, the  $S_2$  GSB signal is observed at 520 nm for spheroidene and at 540 nm for rhodopin glucoside. This shift is mostly due to the longer conjugation length of rhodopin glucoside ( $N = 11$ ) as compared to spheroidene ( $N = 10$ ). However, the exact position of the  $S_2$  peak in the 2D spectrum depends on the spectrum of the excitation pulse, because the Cars are not excited at the absorption maximum but rather at the red shoulder of the  $S_2$  band (Fig. 3E).

The second notable contribution to these 2D spectra is the off-diagonal negative signal of the



**Fig. 3. Absorptive 2D spectra of (A and B) spheroidene measured over a range from 0 to 400 fs, with 2-fs steps, and (C and D) rhodopin glucoside measured over a range from 0 to 400 fs, with 5-fs steps.** Cars were dissolved in acetone. The  $t_2$  times of each spectrum are shown at the top. (E) Absorption spectra/excitation pulse spectra of spheroidene (red dashed/solid lines), and rhodopin glucoside (blue dashed/solid lines).



$S_1$  ESA. A significant feature of that ESA signal is its elongation at longer excitation wavelengths: A substantial shoulder of the ESA is present at 560-nm excitation wavelength in Fig. 3A, and a separate negative peak can be observed at 570- to 580-nm excitation wavelengths in Fig. 3, C and D. The ESA signals at these longer wavelengths indicate an additional state (the X state) from which the excitation energy is transferred to the  $S_1$  state, giving rise to the  $S_1$  ESA.

Even firmer evidence for the X state is the diagonal peak and the cross-peak in Fig. 3B. We found this signal to be very weak and observed only at several  $t_2$  delay times, whereas at all other  $t_2$  delays, it is obscured by  $S_1$  ESA (see movies S3 and S4 for 2D spectra at all  $t_2$  delay times). The absence of the X bleach signal in rhodopin glucoside is due to the different electronic properties of the two molecules. As shown in Fig. 3E, the  $S_2$  absorption band of rhodopin glucoside is not only shifted more to the red but is also broader. As a result, the overlap of the laser pulse spectrum and Car absorption spectrum is larger in rhodopin glucoside. In our experiments, the

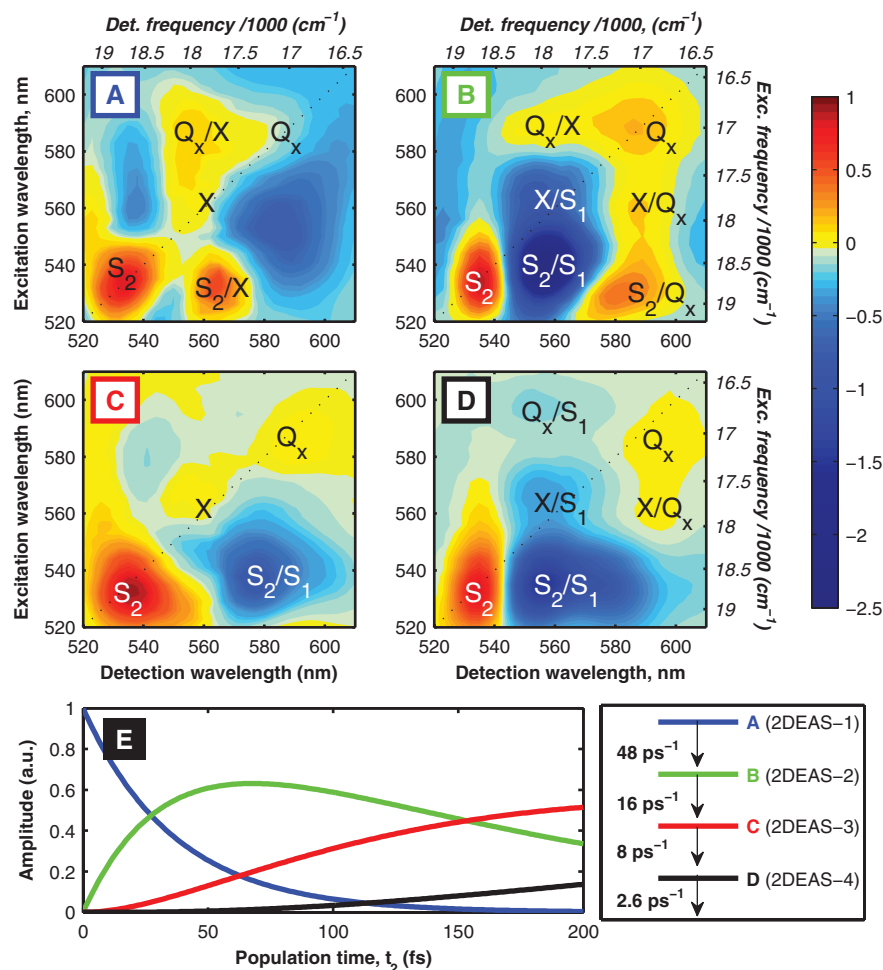
Cars were specifically excited at the very edge of the  $S_2$  absorption in order to minimize signals arising after  $S_2$  excitation (Fig. 3E). No diagonal X signal, or corresponding ESA signals, were observed when the  $S_2$  was excited more to the blue ( $\lambda_{\text{exc}} - \lambda_{S_2} < 50$  nm for spheroidene and  $< 60$  nm for rhodopin glucoside).

Global target analysis is a well-established approach for kinetics-based analysis of time-resolved spectra, usually fluorescence or pump-probe data (24–26). It involves deconvolution of spectral signatures of interconverting components (e.g., populations of excited electronic states) connected to a kinetics model. We report here a global target analysis of the 2D data recorded for *Rps. acidophila*, shown in Fig. 2B. Details of the analysis procedures are described in (21). In the target analysis for 2D spectra, we find how spectral features evolve globally (i.e., across the 2D spectrum as a function of  $t_2$  time) according to the kinetic scheme. Cross-peaks provide strong constraints on the fitting, because cross-peaks contain information on the history of populations (i.e., how they interconvert).

We found that a sequential kinetic scheme for the population dynamics of each kinetic component yielded the most reasonable results [see (21) for discussion]. The features observed in the raw 2D spectra are a product of the overlap of coherent oscillatory amplitude and incoherent relaxation signals. The coherent signals arise from the broadband excitation and can obscure spectral features by modulating the amplitude of bleach and excited-state absorption. Applying a global target analysis to the 2D data required separation of the coherent and incoherent signals. The oscillatory evolution was calculated separately by simulating the experimental Fourier spectra (fig. S9B). We then self-consistently fitted the damped oscillations and the population kinetics to the experimental 2D data. Selected experimental data as a function of population time, together with the corresponding traces obtained after global analysis, are shown in fig. S12.

The results of the global target analysis are presented in terms of population dynamics (Fig. 4E) and their amplitudes in 2D space: The 2D evolutionary-associated spectra (2DEAS) shown in Fig. 4, A to D. The term “evolutionary-associated spectra” means we assumed a sequential kinetic scheme. Owing to the complex kinetics and close values of some of the relaxation rates, each component (species) in this evolutionary kinetic model can be a compartment of several physical states, combined according to the similarity of their temporal properties. Nevertheless, this model allows elucidation of the main features in the 2D spectra and a quantitative estimate of the underlying dynamics. Here we highlight our principal results, whereas the detailed assignment of the 2DEAS spectra can be found in (21), where we also discuss our rationale behind our choice of the scheme and its limitations.

Four species were found to yield a good fit with 2DEAS that correspond most closely to spectral indicators of the Car and BChl states that are interacting by energy transfer and radiationless transitions. The main signals are due to three Car states  $S_2$ , X, and  $S_1$  and one BChl  $Q_x$  state. The  $S_2$  and  $S_1$  features have the strongest amplitude, owing to strong transition dipole moments of the corresponding transitions. The features of the X and  $Q_x$  states have much lower intensity, but they can be observed in all four 2DEAS. The most evident signals of the intermediate X state are the bleach features on the diagonal of the 2DEAS-1 and -3 spectra (marked as X in Fig. 4, A and C). These bleach signals indicate depopulation of the ground state and are present in all 2DEAS. However, in 2DEAS-2 and -4 they are obscured by the negative  $S_1$  ESA signal (21). Rather more informative are the cross-peaks at 560-nm excitation or detection wavelengths. In 2DEAS-1, a strong  $S_2/X$  cross-peak is observed (Fig. 4A). This cross-peak is due to SE from the X state after  $S_2 \rightarrow X$  energy transfer. This process has been previously observed in the stationary fluorescence spectra and transient absorption spectra of isolated Cars (27) and agrees



**Fig. 4. 2DEAS of *Rps. acidophila* (A to D) and corresponding population dynamics (E).** The kinetic scheme and rates are shown to the right of the population kinetics plot. The diagonal peaks are noted by a single symbol designating the corresponding state; the cross-peaks are indicated by two symbols, the first of which represents the locally excited state and the second of which represents an acceptor state.

well with our results. This ultrafast energy transfer ( $>48\text{ ps}^{-1}$  rate) leads to efficient population of the X state. That process is followed by further energy transfer from the X state to lower excited states as indicated in the 2DEAS-2 and -4 spectra (Fig. 4, B and D). Here the negative  $X/S_1$  signal corresponds to the  $X \rightarrow S_1$  energy transfer, whereas the positive  $X/Q_x$  feature is due to Car-to-BChl energy transfer. These energy transfer pathways point to the important role of the intermediate X state in the photoinduced dynamics of the LH2 protein.

Another interesting result is the presence of the negative  $S_1$  ESA signal in 2DEAS-2 and -3. Conventional transient absorption spectroscopy reveals a  $\sim 350\text{-fs}$  lifetime of the hot  $S_1$  state (11, 28, 29), which is well in agreement with the 2DEAS-4 (Fig. 4D). The observation of the  $S_1$  ESA on earlier time scales might be due to higher vibrational levels of the  $S_1$  state. Whether these  $S_{1,v}$  vibrational levels are different, or the same level, which mixes into both 2DEAS-2 and -3, is not clear, and further studies are necessary to answer that question. Consideration of the X state and higher-lying vibrational levels of the  $S_1$  state below the  $S_2$  state might explain the deviation of the  $S_2$  relaxation rate from the energy gap law, observed in several studies of Cars (30, 31).

The data we have reported here clearly demonstrate the presence of a weakly allowed transition (the X state) in both isolated Cars and in LH2 proteins. The global analysis confirmed that the X feature is not a product of the overlap of coherent oscillations with signals from  $S_2$  or  $S_1$  states, but is a separate state that decays on an ultrafast time scale. One of the possible origins of the X state is a higher-energy vibrational level (third or fourth level in the vibrational progression) of the  $S_1$  state. However, in that case, no bleach signal should be observed after excitation of the X state, because the GSB from the X state is weaker than the  $S_1$  ESA signal, as follows from 2DEAS-2 and -4. In contrast, a clear diagonal bleach peak is observed in 2DEAS-1 and -3. Therefore, a much more reasonable assignment of the X state is to the  $IB_u^-$  state, predicted by theoretical calculations to be located below the  $S_2$  state for Cars with conjugation length  $\geq 9$  (5, 32). As a consequence of symmetry properties, the ground state-to- $IB_u^-$  state transition is optically forbidden. In some molecules, transitions to the lower optically forbidden electronic state can borrow intensity from higher optically allowed electronic states via nontotally symmetric vibrations according to the Herzberg-Teller mechanism (33–35). This process of borrowing dipole strength can lead to a weak  $IB_u^-$  signal in Cars.

The intermediate Car dark state ( $IB_u^-$  or  $S_x$  state) was proposed in several experimental studies (6, 27, 36, 37). These reports were based on excited-state absorption signals in pump-probe spectra. However, the assignment of the ESA signals was questioned in later studies. It was shown in several specially designed experiments that the ESA signals can be confused with non-

linear excitation effects, such as two-photon absorption or impulsive Raman scattering (7, 8, 38). Thus, until now, the existence of the  $IB_u^-$  state remained controversial. In contrast to previous reports, in this work we observed the  $IB_u^-$  state via its GSB signal. Because a GSB signal gives the energetic position of the excited state relative to the ground state, this observation is compelling evidence of the presence of the  $IB_u^-$  state.

In 2002, Cerullo *et al.* suggested that the intermediate Car state, located between the Car  $S_2$  and BChl  $Q_x$  states, plays an important role in light-harvesting proteins (6). The clearest indication of the contribution of the  $IB_u^-$  state is the disagreement between theoretically calculated and experimentally measured efficiencies of Car-to-BChl energy transfer in LH2 complexes. The calculations performed on the LH2 complex of *Rps. acidophila* took into account the main features of the BChl and Car molecules by precise description of the size and shape of their transition densities (16). This method allowed the calculation of electronic couplings and energy transfer rates with high precision; nevertheless, the obtained values were less than half the experimentally measured values (9–12). This deviation was explained by a large energy gap and small spectral overlap between the  $S_2$  and  $Q_x$  states. The cross-peaks observed in 2D spectra in the current work (Fig. 2 and figs. S2 to S5) clearly show evidence for electronic coupling between the  $IB_u^-$  state and both Car  $S_2$  and BChl  $Q_x$  states. This coupling increases the spectral overlap and therefore the Car-BChl energy transfer efficiency. The present study suggests that the disagreement in energy transfer efficiencies is due to the absence in the theoretical model of the  $IB_u^-$  state, which closes the energy gap and promotes Car-to-BChl energy transfer.

The energy transfer pathways within light-harvesting proteins critically depend on the molecular structure of the interacting chromophores as well as their orientation within the protein. In this work, the presence of the Car intermediate state has been demonstrated for two LH2 proteins, containing Cars with 10 and 11 conjugated C=C double bonds. Therefore, thorough studies (both theoretical and experimental) are necessary to revise the understanding of interaction between Cars and Chls. In this respect, 2D electronic spectroscopy in combination with global analysis has proven to be a preeminent tool for studying complex multichromophore systems, providing insights into the energy conversion processes and revealing spectral properties invisible to other techniques.

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#### Supplementary Materials

www.sciencemag.org/cgi/content/full/340/6128/52/DC1  
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# Evidence for a Symmetrical Fluoronium Ion in Solution

Mark D. Struble, Michael T. Scerba, Maxime Siegler, Thomas Lectka\*

Halonium ions, in which formally positively charged halogens (chlorine, bromine, and iodine) are equivalently attached to two carbon atoms through three-center bonds, are well established in the synthetic chemistry of organochlorides, bromides, and iodides. Mechanistic studies of these ions have generated numerous insights into the origins of stereoselectivity in addition and displacement reactions. However, it has not been clear whether fluorine can form a halonium ion in the same manner. We present chemical and theoretical evidence for the transient generation of a true symmetrical fluoronium ion in solution from an appropriately configured precursor.

**H**alonium ions are organic compounds in which formally positively charged halogens (Cl, Br, and I) are equivalently attached to two carbon atoms through three-center bonds (Fig. 1). Since the 1930s, halonium ions have been known to be a great source for unique synthetic pathways and insight into reaction mechanisms (1, 2). Iodonium ions are relatively stable, often isolable, and synthetically useful as reagents (3). Bromonium ions are generally transient, occasionally isolable, but nonetheless well-documented intermediates in dibromination and various bromofunctionalization reactions, often taking the form of bromacyclopropane rings (4). Chloronium ions are somewhat less well documented, although Stoyanov, Reed, and co-workers have recently synthesized some remarkably stable examples (5).

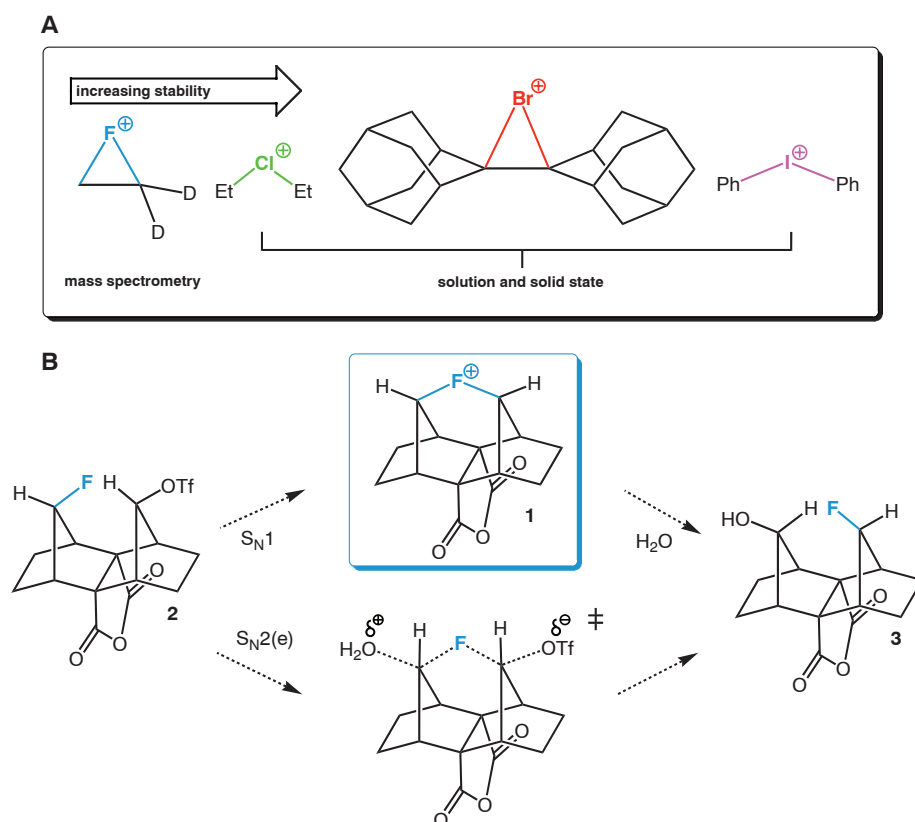
In contrast, the best evidence to date for a fluoronium ion has been obtained in the gas phase as a result of a neutral products analysis of a mass spectrometry experiment that showed conclusive fluorine shifts (Fig. 1A) (6). Several reasons may explain the absence of such compounds in solution, not the least of which is fluorine's high electronegativity, which lowers the propensity for sharing its lone pairs of electrons through bridging. Perhaps the closest a lab has come to the synthesis of a fluoronium ion in solution stems from the work of Gabbai, who generated a cation in which a C-F bond of normal length engages in an electrostatic interaction (C-F distance 2.44 Å) with a positively charged center (7).

In any case, symmetrical fluoronium ions in solution represent a major missing link in halonium ion chemistry. In light of fluorine's ability to impart many unusual and beneficial properties to organic compounds (such as its ability to fundamentally alter a drug's therapeutic properties), information on the mechanisms through which it reacts is of great interest (8). A fluoronium ion would also represent a long-sought goal in synthetic and mechanistic organic chemistry, as it would demonstrate that fluorine can react through halonium ion pathways. Here, we provide strong evidence, based on isotopic

labeling studies, control experiments, stereochemistry, and calculations, for the transient generation of fluoronium ion **1** (Fig. 1B) in solution from an ideally configured precursor.

Given fluorine's electronegativity and limited capacity for hypervalent bonding, fluoronium ion detection rests critically on the nature of the system, which must favor symmetrical ion formation to the exclusion of all other reasonable possibilities. We deemed a hydrolytically labile precursor molecule such as **2** (Fig. 1B) to be suitable for the following reasons: (i) It has a rigid, cage-like framework in which F resides in close proximity to an electrophilic carbon bearing a reactive triflate (OTf) leaving group that should lead through a low-energy, least-motion pathway to ion **1**. In a recent publication, we synthesized

cage compounds in which fluorine interacts closely with a C=C or C-H bond (9). In the case of the present system, we aimed to bring F into even closer proximity to the opposing bridge in order to promote fluoronium ion formation. For example, the optimized structure of **2** at density functional theory (DFT) level B3LYP/6-311++G\*\* and Møller-Plesset level MP2/6-311++G\*\* reveals a close interaction of the inner fluorine and C of the opposite bridge of 2.78 Å. (ii) Carbocationic rearrangements of **1** should be suppressed by the fact that virtually all reasonable isomeric competitors are higher in energy. (iii) Hydrolysis of precursor **2** is likely to occur through a cationic  $S_N1$  mechanism rather than an extended  $S_N2$  mechanism [ $S_N2(e)$ ], as a result of both fluorine's poor leaving-group ability and steric hindrance to nucleophilic attack (Fig. 1B) (10). For example, 7-norbornyl substrates are known to undergo  $S_N1$  solvolysis, in contrast to most other secondary systems, by virtue of nonclassical ion formation with attendant steric blockage of backside attack (11, 12). The  $S_N2(e)$  pathway is expected to be very strained by virtue of nonbonded interactions of the nucleophile and leaving group with the proximate two one-carbon bridges (in the manner of the 7-norbornyl system). As can be seen in models, the trajectory for approach of a nucleophile to the backside of the C-F bond is nonoptimal. On the other hand, the  $S_N1$  reaction to form the fluoronium **1** is expected to release a substantial amount of strain energy.



**Fig. 1. Milestones in halonium ion chemistry.** (A) Observed halonium ions from references (6), (5), (4), and (3), respectively. (B) The approach to fluoronium ion **1**.

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Ion **1** is predicted to be a stable,  $C_{2v}$ -symmetric minimum at suitable levels of theory [HF, MP2, DFT (B3LYP, EDF1, EDF2, PBEPBE)], all employing the 6-311++G\*\* and cc-pVTZ basis sets), even in the presence of various dielectric constants and solvent models (13). Competing asymmetric “nonfluoronium” forms (especially those that would result from simple ionization of the precursor) are not found to be stable according to acceptable HF-, MP2-, and DFT-based levels of theory. Theoretical predictions of carbocation geometries are very often definitive, as they are closed-shell species with a reduced electron correlation problem (14). The fluoronium structure results from any number of asymmetric starting geometries in which C-F bonds or interactions are inequivalent. An intramolecular hydride shift to form an undesired but stable  $\alpha$ -fluoro cation (a process that plagues nonrigid precursors) is stereo-electronically precluded.

The synthetic route to the precursor molecule **2** is shown in Fig. 2. The first step was the reaction of the known dienophile **4** with silylated diene **5** under high pressure (a similar reaction, conducted under heating at 1 bar, produced no desired products) (9, 15). This results in a complex mixture of diastereomers from which **6** could be separated by careful column chromatography in 23% yield. Alkene **6** was susceptible to desilylation and was rapidly carried forward through Fleming-Tamao oxidation and reduced with a palladium/carbon catalyst under hydrogen at 2 bar to afford alcohol **7** in 92% yield for the two steps (16). The mostly through-space coupling ( $^{13}\text{C}$ - $^{19}\text{F}$ ) of the opposing atoms [ $\text{R}_2\text{CH}^{19}\text{F}$  and  $\text{R}_2(\text{OH})\text{H}^{13}\text{C}$ ] in the nuclear magnetic resonance (NMR) spectrum of **7** is 48 Hz (calculated at B3LYP/6-311++G\*\* to be 48 Hz as well; the one-bond C-F coupling is 212 Hz), indicating substantial interaction between the bridges (17–19); coupling constant calculations were performed on the Gaussian 09 and Spartan '06 programs (13, 20, 21).

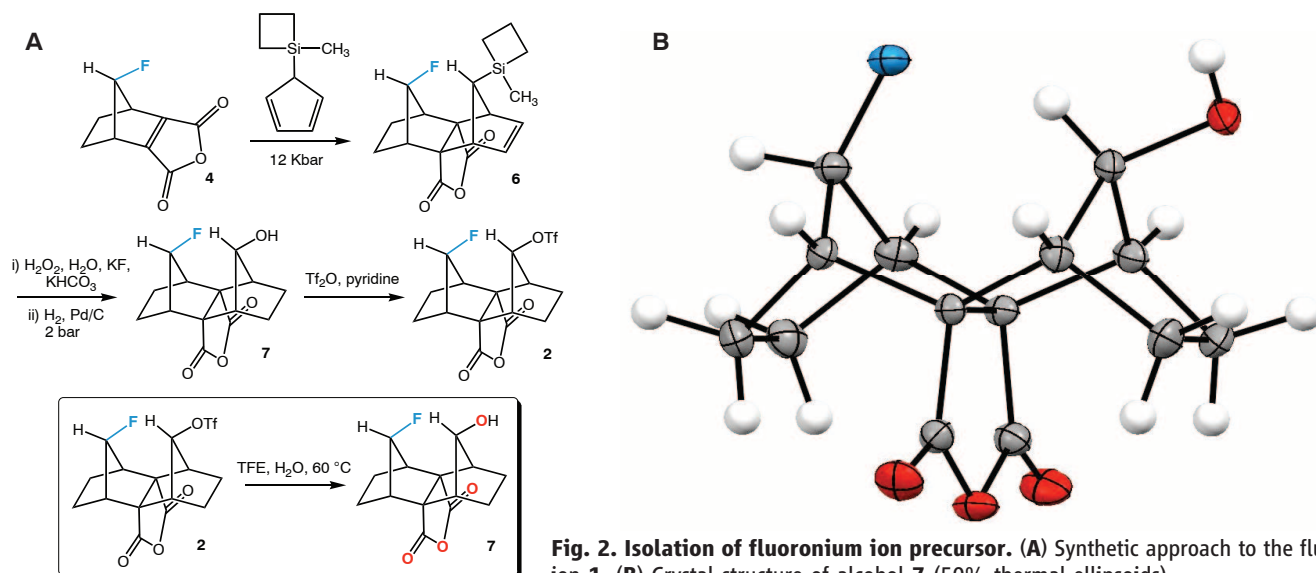
We also obtained a crystal structure of the precursor alcohol **7** in which the fluorine atom seems poised to attack the opposite bridge given an opportunity (C-F distance 2.83 Å; note agreement with calculation: 2.83 Å at B3LYP/6-311++G\*\*). Consequently, the strain with which the bridges repel each other should be relieved during cation formation (Fig. 2B). The alcohol **7** was converted to **2** under standard conditions ( $\text{TiF}_4$ , pyridine, room temperature). Crude triflate **2** was partitioned between methylene chloride and water, which yielded a fairly pure sample (>96% by NMR) that was quickly characterized by  $^{19}\text{F}$  and  $^1\text{H}$  NMR spectroscopy because of its tendency to decay over time. The triflate was then dissolved in 2,2,2-trifluoroethanol (TFE) in the presence of variable quantities of water (v/v). Smooth reaction, over 6 to 10 hours at 60°C, converted triflate **2** back to the starting alcohol (and small amounts of the corresponding 2,2,2-trifluoroethyl ether) cleanly and in high yield (96% after work-up and isolation; Fig. 2A). To our gratification, no measurable quantities of skeletally rearranged or defluorinated products were detected by  $^{19}\text{F}$  or  $^1\text{H}$  NMR, in accord with our prediction of a fairly simple potential energy surface for the reaction of this gated system.

However, the fundamental problem with triflate **2** is that its reaction with water through either mechanism gives rise to only one identifiable product, namely, the starting alcohol. No means to differentiate between the extended  $\text{S}_{\text{N}}2(\text{e})$  substitution leading to inversion and an  $\text{S}_{\text{N}}1$  reaction involving fluorine assistance (associated with the fluoronium ion) would be evident. Nucleophilic trapping of a fluoronium intermediate from either one-carbon bridge yields the very same product that is expected for the hypothetical  $\text{S}_{\text{N}}2(\text{e})$  process. For completeness, one other less plausible alternative should be mentioned: an  $\text{S}_{\text{N}}1$ -type reaction with retention. In this case, fluorine may act as an anchimeric assistant, without full fluoronium ion formation, or else simply

blocks backside attack. There exist examples in the literature in which hydrogen may play such an anchimeric role (22).

At this point we sought to synthesize a system in which a minor isotopic perturbation would render the precursor spectroscopically and stereochemically asymmetric, thus allowing us to differentiate between the major mechanistic possibilities. In fact, it was precisely stereochemical evidence that helped establish the existence of bromonium ions (23). One way to do this without fundamentally altering the electronic nature of the fluoronium ion itself is through a remote isotopic substitution on a two-carbon bridge. Such a substitution presumably gives rise to potential  $\gamma$ -deuterium kinetic isotope effects; in a sterically and electronically similar case, the solvolysis of 7-norbornyl triflates, small positive  $\gamma$ -deuterium isotope effects were observed ( $k_{\text{H}}/k_{\text{D}} = 1.024$  to 1.038). These were interpreted as being steric effects that originate during departure of the leaving group (12). As we do not compare labeled and unlabeled isomeric substrates, this question does not apply to our system. On the other hand, a small steric isotope effect on product distribution may occur, although it would be within the error limits in which we quantified our results, and would favor slight retention over inversion.

The synthesis of the labeled isomer was easily accomplished by Fleming-Tamao oxidation of anhydride **6** followed by diastereoselective reduction with dideuteriodiimide (67% yield) and triflation to afford **2-2d** (deuterium incorporation is >97% by mass spectrometry; Fig. 3) (24). In this case, trapping of a fluoronium ion (generated by the classical  $\text{S}_{\text{N}}1$  process) by water should give rise to two labeled isomeric alcohols in comparable amounts. With the labeled triflate isomer in hand, we monitored its conversion back to alcohol **7-2d** in a solution of 20% water in TFE v/v at 60°C [to minimize autocatalysis, we added 1.5 equivalents (eq.) of the weak base 2,6-lutidine to absorb the generated triflic acid] at



**Fig. 2. Isolation of fluoronium ion precursor.** (A) Synthetic approach to the fluoronium ion **1**. (B) Crystal structure of alcohol **7** (50% thermal ellipsoids).



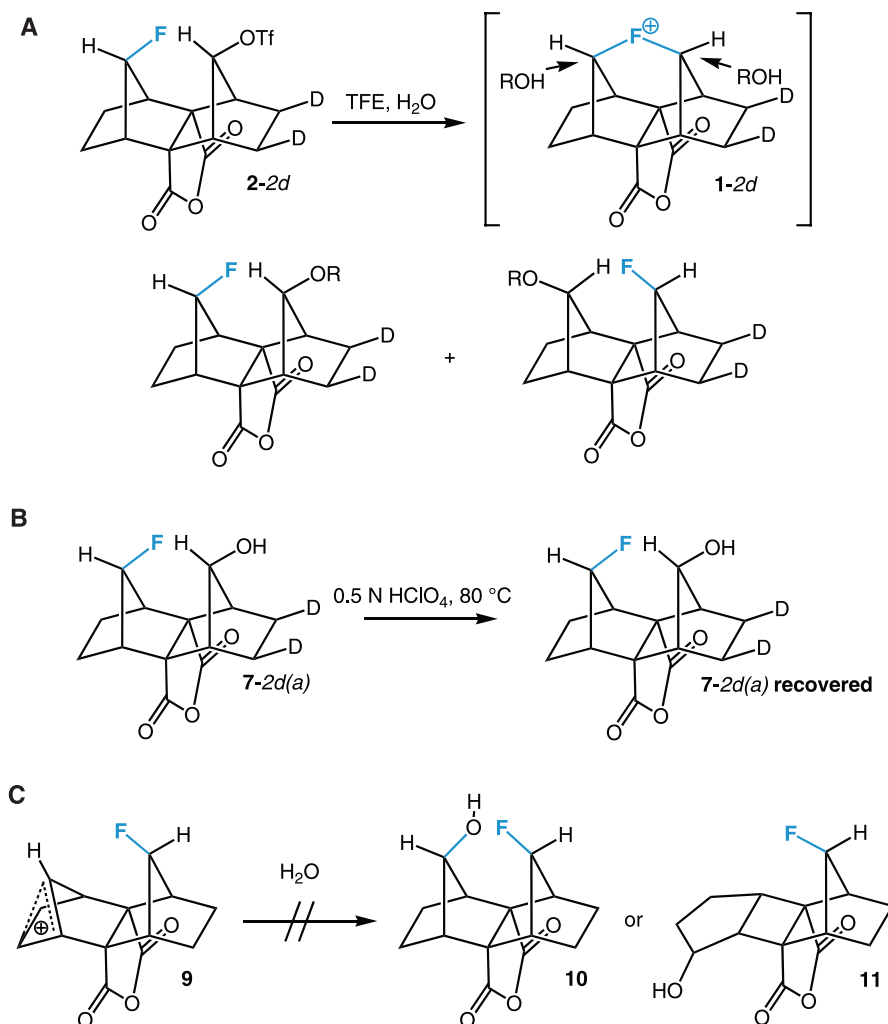
60°C (25). The reaction provided a 1:1 mixture of labeled isomers **7-2d(a)** and **7-2d(b)** in 96% yield, along with a small amount of a 1:1 mixture

of the corresponding TFE ethers, **8-2d(a)** and **8-2d(b)** (26). Labeled isomers were quantified by integration of  $^1\text{H}$  spectra and of isotope-shifted,

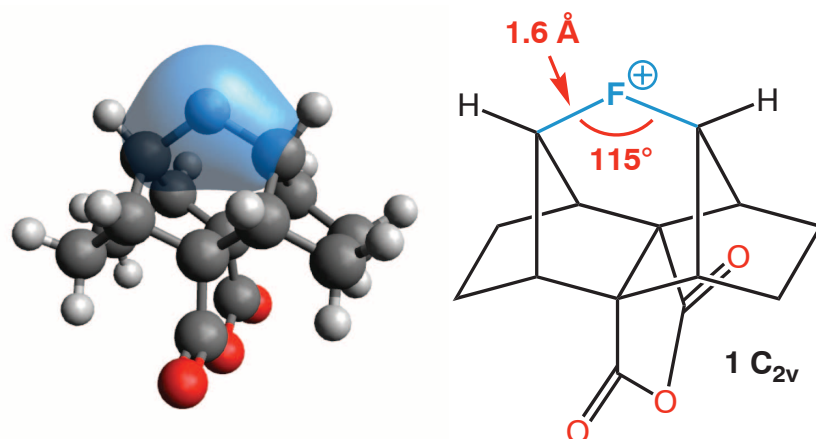
split  $^{13}\text{C}$  NMR peaks. Experimental and calculated chemical shifts were found to be in good agreement for both  $^{19}\text{F}$  and  $^{13}\text{C}$  (13, 27). When an analogous experiment was performed in pure TFE (60°C, 1.5 eq. 2,6-lutidine), 99% of the TFE ether isomers, **8-2d(a)** and **8-2d(b)**, also in a 1:1 ratio, were obtained.

Evidence for the generation of a fluoronium ion in solution is as follows: (i) The equivalent ratio of labeled isomers **7-2d** (and **8-2d** in pure TFE) is consistent with a scenario in which the predominant solvolytic pathway results from nucleophilic attack on either bridge of a symmetrical ionic intermediate in which counterions do not interfere (Fig. 3A). (ii) The conditions of the reaction are consistent with what we would expect for the  $\text{S}_{\text{N}}1$  process (relatively non-nucleophilic medium and high dielectric constant) (28). Schneider and Schmidt have used 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to observe pure  $\text{S}_{\text{N}}1$  reactions on cyclohexyl tosylates (29); we performed a hydrolysis in similar conditions, but results were less accurate because of difficulties experienced in the integration of small quantities of the product alcohols. (iii) A Grunwald-Winstein kinetic analysis using  $\text{Y}_{\text{OTf}}$  parameters yielded an  $m$  value of 1.54, indicating a  $\text{S}_{\text{N}}1$  reaction, as expected ( $m$  values that trend  $>1$  indicate a solvent response indicative of an  $\text{S}_{\text{N}}1$  reaction, whereas  $m$  values of 0.25 to 0.35 indicate  $\text{S}_{\text{N}}2$  reactions) (30). (iv) Alternative pathways—including the  $\text{S}_{\text{N}}2(\text{e})$  reaction, the  $\text{S}_{\text{N}}1$  reaction with retention, or an unsymmetrical ion-pairing effect in the  $\text{S}_{\text{N}}1$  reaction—operate only to a very small extent, if at all. The fact that strictly 1:1 ratios of labeled isomeric products were obtained in various mixtures of water and cosolvents argues for a pure  $\text{S}_{\text{N}}1$  reaction as well. (v) As stated, ion **1** is computationally stable at all suitable levels of theory, whereas the conceptual nonfluoronium (asymmetric or nonbridging) isomer, with all its attendant strain and steric hindrance to nucleophile trapping, is not stable at any suitable level of theory.

Another eventuality to rule out in order to support the case concerns the possibility that sequential  $\text{S}_{\text{N}}2(\text{e})$  reactions occur after hydrolysis that scramble the labeled isomeric positions through oxonium intermediates. The inclusion of 2,6-lutidine was in part meant to suppress this potential process; however a control experiment, under vigorous conditions, was performed as confirmation. Heating labeled isomer **7-2d(a)** in 0.5 N perchloric acid at 80°C for 6 hours resulted in virtually quantitative re-isolation of unrearranged substrate (Fig. 3B); note that the sterically encumbered anhydride group of **7** and related molecules is virtually inert to hydrolysis. On the other hand, imagine that a hypothetical, non-fluoronium alternative could exist. A one-carbon bridge could be pushed back from an interaction with F, toward the two-carbon bridge (structure **9**), in a manner analogous to that observed in the 7-norbornyl cation or the skeletally similar *exo-exo*-5,8-dimethanonaphthalene system (12, 31).



**Fig. 3. Solvolysis experiments.** (A) Labeling study as test for ion **1** formation. Reaction with 20% (v/v)  $\text{H}_2\text{O}$  in TFE affords a 1:1 mixture of isomers **7-2d(a)** and **7-2d(b)** ( $\text{R} = \text{H}$ ). Reaction with pure TFE affords a 1:1 mixture of **8-2d(a)** and **8-2d(b)** ( $\text{R} = \text{CH}_2\text{CF}_3$ ). (B) Control experiment to rule out sequential  $\text{S}_{\text{N}}2$  reactions. (C) Hypothesized outcome of unsymmetrical ion trapping.



**Fig. 4.**  $\sigma$ -Molecular orbital of the three-center C-F-C bond (0.02 isosurface value,  $-1.31$  eV) and structure of **1** at B3LYP/6-311++G\*\*.

Such an entity may trap water from the inner position, resulting in alcohol **10**, or in a rearranged product, namely cyclobutane **11**. Neither **10** nor **11** (nor derived labeled isomers) are observed in the reaction (Fig. 3C).

What is the nature of the three-center bond in putative fluoronium ion **1**? According to calculations, much of the positive charge is sustained by adjacent carbons and hydrogen atoms (although fluorine itself is considerably more positively charged than in a typical C-F bond). For example, the two C-F bond distances in **1** are equivalent (B3LYP/6-311++G\*\*, 1.60 Å; MP2/6-311++G\*\*, 1.57 Å; PBEPBE/6-311++G\*\*, 1.61 Å) and allow hypervalent fluorine to position itself snugly within a  $C_{2v}$  symmetric polycyclic cage composed of interlocking five- and six-membered carbocyclic rings. The C-F-C bond angle is  $115^\circ$  in the former calculations ( $114^\circ$  at PBEPBE), permitting F to fit optimally within the lattice of the cage, and associated electron pairs to maximize repulsion. Figure 4 depicts the filled, very low-lying ( $-1.31$  eV), delocalized  $\sigma$  orbital that is primarily responsible for three-center C-F-C bonding. Why would ion **1** trap solvent in such a clean fashion rather than decompose? A calculated barrier to unimolecular decomposition may provide a clue. Electron impact mass spectrometry of **1** shows a fragment corresponding to the cyclopent-2-en-1-yl cation, a resonance-stabilized allylic system. The loss of this species through a calculated barrier of 24.5 kcal/mol (B3LYP/6-311++G\*\*) defines a potential low-energy pathway to unimolecular decomposition. It is thus justifiable to conclude that nucleophilic trapping of **1** would be much faster.

Our study points to fluorine's potential role as an anchimeric assistant to open up possibilities for new reactions that can capitalize on a fluoronium intermediate to control regioselectivity, diastereoselectivity, and overall reactivity in organofluorinated molecules. Fundamental insights into the mechanisms by which fluorine reacts in organic molecules are of great importance to several fields, especially synthetic and medicinal chemistry.

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#### Supplementary Materials

www.sciencemag.org/cgi/content/full/340/6128/57/DC1  
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Data Files S1 to S29  
References (32–35)

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## Photochemical Route for Accessing Amorphous Metal Oxide Materials for Water Oxidation Catalysis

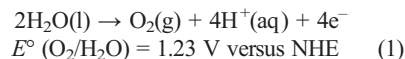
Rodney D. L. Smith, Mathieu S. Prévot, Randal D. Fagan, Zhipan Zhang, Pavel A. Sedach, Man Kit Jack Siu, Simon Trudel,\* Curtis P. Berlinguette\*

Large-scale electrolysis of water for hydrogen generation requires better catalysts to lower the kinetic barriers associated with the oxygen evolution reaction (OER). Although most OER catalysts are based on crystalline mixed-metal oxides, high activities can also be achieved with amorphous phases. Methods for producing amorphous materials, however, are not typically amenable to mixed-metal compositions. We demonstrate that a low-temperature process, photochemical metal-organic deposition, can produce amorphous (mixed) metal oxide films for OER catalysis. The films contain a homogeneous distribution of metals with compositions that can be accurately controlled. The catalytic properties of amorphous iron oxide prepared with this technique are superior to those of hematite, whereas the catalytic properties of  $\alpha\text{-Fe}_{100-y-z}\text{Co}_y\text{Ni}_z\text{O}_x$  are comparable to those of noble metal oxide catalysts currently used in commercial electrolyzers.

The scalable storage of renewable energy by means of converting water to hydrogen fuels ( $\text{H}_2$ ) electrochemically hinges on fundamental improvements in catalytic materials. A large overpotential ( $\eta$ ) is usually re-

quired to produce  $\text{H}_2$  at a practical rate, which is primarily the result of slow oxygen ( $\text{O}_2$ ) evolution kinetics. Despite recent advances in the development of heterogeneous catalysts to negotiate the OER (Eq. 1;  $E^\circ$  is the standard potential and

NHE is the normal hydrogen electrode) (*1–4*), substantial market penetration by commercial electrolyzers has been hindered by the absence of inexpensive catalytic materials that exhibit high current densities ( $j$ ) ( $>0.5$  A  $\text{cm}^{-2}$ ) at low  $\eta$  ( $<0.3$  V) over prolonged time periods.



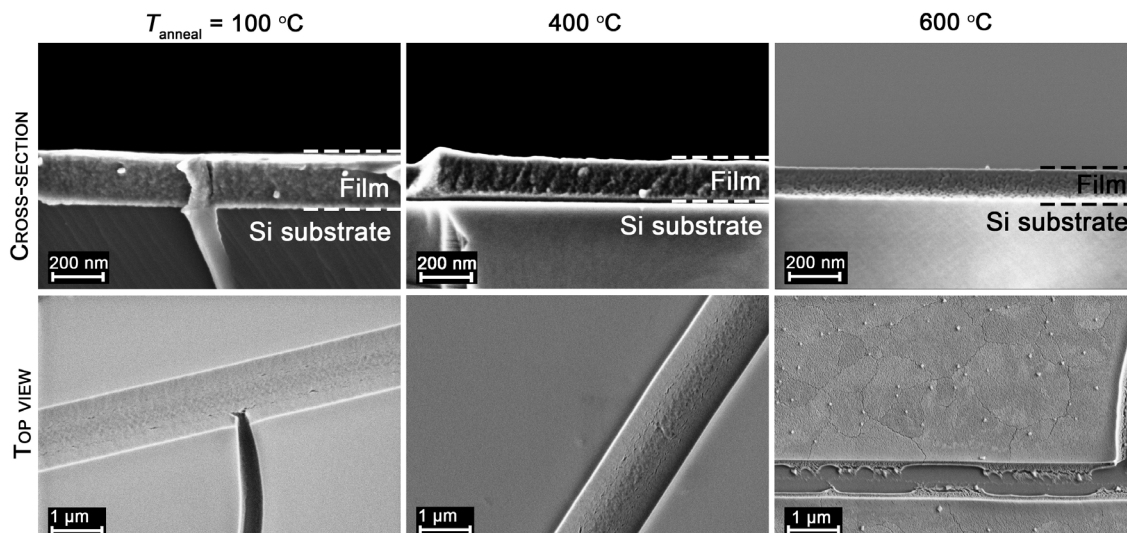
Metal oxides are the most durable and active water oxidation catalysts (*1, 2*). Although  $\text{IrO}_2$  and  $\text{RuO}_2$  are among the best OER catalysts (*5, 6*), a myriad of perovskite (*3, 7*) and spinel (*8*) solids have proven to be competent catalysts. In recent years, amorphous metal oxides have also been demonstrated to be excellent OER catalysts (*4, 9*), including when integrated with photoactive electrodes (*10–12*). Although an increasing number of amorphous metal oxide catalysts have been reported (*4, 9, 13–17*), most have been

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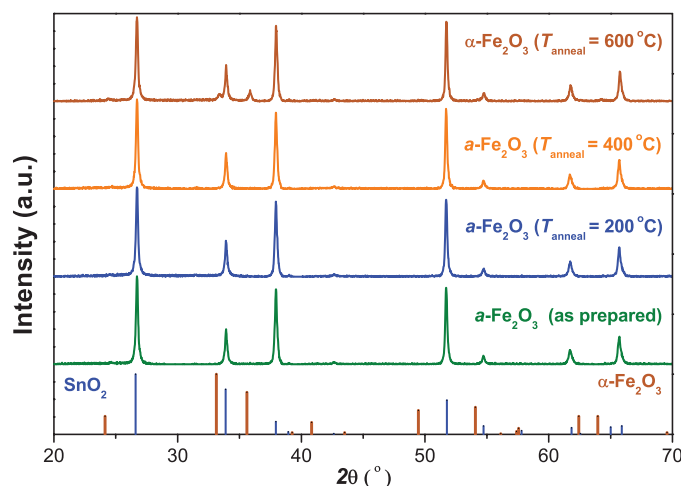
**Fig. 1.** SEM cross-section and top-down surface images of  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub> films prepared by PMOD, followed by a 1-hour annealing step in air at  $T_{\text{anneal}} = 100^\circ$ ,  $400^\circ$ , or  $600^\circ\text{C}$ .



produced by electrodeposition techniques—a methodology that does not necessarily translate to every metal. Moreover, amorphous film deposition can be sensitive to the voltage protocol, thereby rendering few examples of amorphous OER catalysts composed of multiple metal identities. Considering that the vast majority of crystalline catalysts consist of more than a single metallic element, there are anticipated benefits to developing amorphous metal oxide catalysts of more complex metal compositions. Following this line of inquiry, we show herein that photochemical metal-organic deposition (PMOD) (18) is a facile technique for preparing amorphous phases of (mixed) metal oxides that generate high OER electrocatalytic activity. This proof-of-principle investigation of amorphous iron oxide ( $\alpha$ -Fe<sub>2</sub>O<sub>3</sub>) films and mixed-metal compositions ( $\alpha$ -Fe<sub>100-x-y</sub>-Co<sub>x</sub>-Ni<sub>y</sub>O<sub>x</sub>) demonstrates the broad applicability of this methodology for accessing new OER catalysts.

Iron(III) 2-ethylhexanoate was used as a PMOD precursor for making  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub> thin films (19). Thin films of optical quality were prepared by spin coating a hexane solution of the precursor onto fluorine-doped tin oxide (FTO) (for electrochemical and spectroscopic characterization) or silicon (for electron microscopy and elemental analyses). These films were then irradiated with 185- and 254-nm light until the vibrational stretching frequencies of the 2-ethylhexanoate ligands could no longer be detected by infrared spectroscopy (fig. S1) (20). The resultant  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub> films were annealed at temperatures ( $T_{\text{anneal}}$ ) up to  $600^\circ\text{C}$ . The thicknesses of the  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub> films were measured by means of cross-sectional scanning electron microscopy (SEM) imaging (Fig. 1). For  $T_{\text{anneal}} \leq 200^\circ\text{C}$ , films had a thickness of 150 to 200 nm; the thickness gradually decreased at progressively higher  $T_{\text{anneal}}$  values, reaching 100 to 150 nm at  $500^\circ$  to  $600^\circ\text{C}$ . Smooth, homogeneous films with only minor microscale defects were observed for  $T_{\text{anneal}} \leq 200^\circ\text{C}$ ; higher  $T_{\text{anneal}}$  led to a greater degree of film cracking and an

**Fig. 2.** XRD powder patterns acquired on as-prepared (i.e., no annealing step) and annealed ( $T_{\text{anneal}}$  indicated) Fe<sub>2</sub>O<sub>3</sub> films. Bragg reflections for hematite are observed for films annealed at  $T_{\text{anneal}} > 500^\circ\text{C}$ . Patterns for hematite [Joint Committee on Powder Diffraction Standards (JCPDS) card 33-664] and SnO<sub>2</sub> (JCPDS card 41-1445) are also shown.

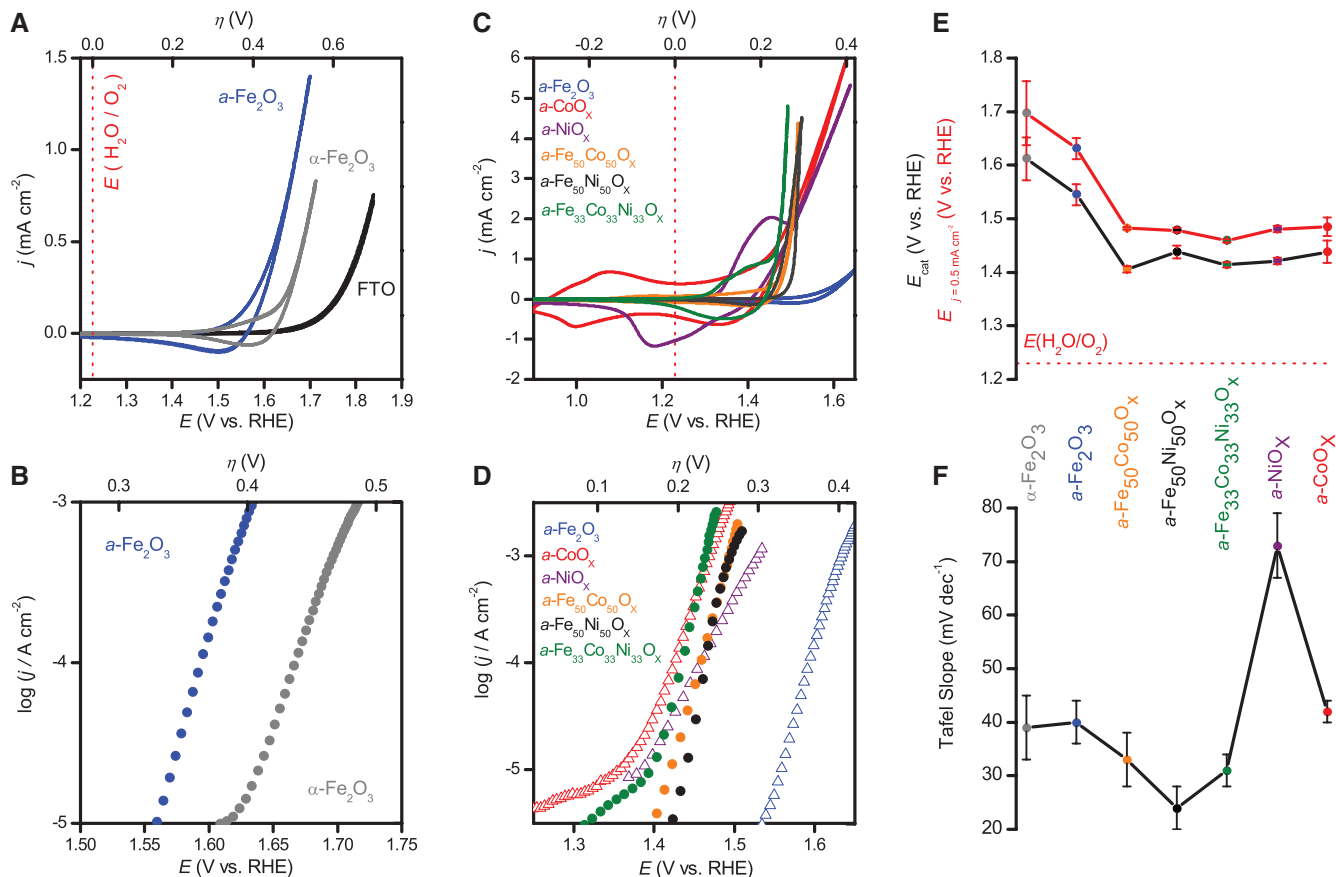


increase in apparent porosity (fig. S2) (20). The greater degree of defects presumably arises upon contraction of the film when transitioning from an amorphous to a crystalline structure.

X-ray diffraction (XRD) (Fig. 2 and fig. S3) (20) revealed no evidence for a crystalline phase at  $T_{\text{anneal}} < 500^\circ\text{C}$ . Films heated to higher temperatures produced diffraction peaks congruent with those of hematite ( $\alpha$ -Fe<sub>2</sub>O<sub>3</sub>). These results are consistent with an extended x-ray absorption fine structure analysis of as-prepared  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub> films (19). Ultraviolet-visible (UV-vis) diffuse reflectance spectra recorded on films subjected to  $T_{\text{anneal}} \geq 500^\circ\text{C}$  revealed optical profiles resembling that of hematite (fig. S4) (20, 21). Films treated at  $T_{\text{anneal}} \leq 200^\circ\text{C}$  rendered spectral features distinctive from that of hematite. Optical data for films annealed at  $300^\circ$  and  $400^\circ\text{C}$  contained features that appear to be a hybrid of the two phases. These collective data show that although the structure of the amorphous material is not sufficiently coherent to produce observable Bragg reflections until  $T_{\text{anneal}} \geq 500^\circ\text{C}$ , the UV-vis data indicate that hematite may begin to form at lower temperatures. Hence, the electro-

chemical data presented below are on films annealed at  $100^\circ\text{C}$  to ensure that the catalytic activity originates exclusively from  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub>.

The electrochemical behavior of  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub> and hematite is shown in Fig. 3, A and B. An oxidative sweep of the crystalline film revealed a small rise in current at 1.50 V versus RHE (reversible hydrogen electrode) that was maintained until a sharp increase in current at  $\sim 1.62$  V, corresponding to catalytic water oxidation. The cyclic voltammogram for  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub> was also featureless until the onset of catalytic water oxidation at  $\sim 1.50$  V. The catalytic wave was independently confirmed to be associated with water oxidation on the basis of O<sub>2</sub> evolution experiments (fig. S5) (20). Steady-state current densities ( $j$ ) as a function of  $\eta$  were recorded to probe the kinetics of the reactions. Once a threshold potential was reached,  $\log(j)$  exhibited a sharp increase and a linear dependence on  $\eta$  where the current density became limited by electron-transfer kinetics at the electrode surface. This experiment reveals useful electrode kinetic metrics, including the onset of linearity ( $E_{\text{cat}}$ ) and the Tafel slope (Fig. 3B and fig. S6) (20).  $E_{\text{cat}}$  was observed at  $1.55 \pm$



**Fig. 3.** Electrochemical behavior for oxide thin films prepared from 15% w/w precursor solutions by the PMOD technique, followed by annealing at 100°C. **(A)** Cyclic voltammograms for films of  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub> (blue) and hematite (gray), and a blank fluorine-doped tin oxide (FTO) substrate. **(B)** Tafel plot showing the higher catalytic activity of  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub> relative to hematite. Comparison of electrochemical behavior for thin films of  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub>,  $\alpha$ -Fe<sub>50</sub>Ni<sub>50</sub>O<sub>x</sub>,  $\alpha$ -Fe<sub>50</sub>Co<sub>50</sub>O<sub>x</sub>, and  $\alpha$ -Fe<sub>33</sub>Co<sub>33</sub>Ni<sub>33</sub>O<sub>x</sub> films; **(C)** cyclic voltammograms; **(D)**

Tafel plots; **(E)** onset potentials ( $E_{cat}$ ) and potentials required to reach  $j = 0.5$  mA cm<sup>-2</sup>; and **(F)** Tafel slopes for the various catalyst films. Electrochemistry conditions: counterelectrode = Pt mesh; reference electrode = Ag/AgCl, KCl(sat'd); scan rate = 10 mV s<sup>-1</sup>; electrolyte = 0.1 M KOH(aq); current densities were corrected for uncompensated resistance. Dashed red lines correspond to the thermodynamic potential for water oxidation (26). Error bars indicate the SD between multiple electrodes (three minimum).

0.02 V ( $\eta = 0.32$  V) for  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub>, corresponding to a 60-mV improvement in  $\eta$  relative to that of hematite. Tafel slopes of  $\sim 40$  mV dec<sup>-1</sup> were observed for both materials, and a current density of 0.5 mA cm<sup>-2</sup> was reached at  $1.63 \pm 0.02$  V for  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub> (compared with  $1.70 \pm 0.06$  V for hematite), thus demonstrating the superior electrocatalytic activity of the amorphous phase relative to the crystalline phase.

We next used PMOD to fabricate amorphous mixed-metal oxides of iron, nickel, and cobalt (19, 22) in pursuit of improved catalysts. Precursor solutions were prepared with the appropriate stoichiometries to produce  $\alpha$ -Fe<sub>50</sub>Co<sub>50</sub>O<sub>x</sub>,  $\alpha$ -Fe<sub>50</sub>Ni<sub>50</sub>O<sub>x</sub>, and  $\alpha$ -Fe<sub>33</sub>Co<sub>33</sub>Ni<sub>33</sub>O<sub>x</sub> upon photolysis (table S1) (20);  $\alpha$ -CoO<sub>x</sub> and  $\alpha$ -NiO<sub>x</sub> films were also prepared by PMOD to serve as benchmark materials. The UV-vis diffuse reflection spectra of the mixed-metal films (fig. S7) (20) reveal poorly resolved absorption features that do not match those of the known crystalline phases of the Ni or Co oxides (23–25). The SEM images of the mixed-metal oxide films (fig. S8) (20) revealed a smooth surface akin to that observed

**Table 1.** Comparison of catalytic parameters of amorphous and crystalline metal oxide OER catalysts.

|                                             | $E_{cat}$<br>(V vs. RHE) | Tafel slope<br>(mV dec <sup>-1</sup> ) | Reference |
|---------------------------------------------|--------------------------|----------------------------------------|-----------|
| $\alpha$ -FeO <sub>x</sub>                  | $1.55 \pm 0.02$          | $40 \pm 4$                             | (27)      |
| $\alpha$ -CoO <sub>x</sub>                  | $1.44 \pm 0.02$          | $42 \pm 2$                             | (27)      |
| $\alpha$ -NiO <sub>x</sub>                  | $1.42 \pm 0.01$          | $73 \pm 6$                             | (27)      |
| $\alpha$ -FeCoO <sub>x</sub>                | $1.41 \pm 0.01$          | $33 \pm 5$                             | (27)      |
| $\alpha$ -FeNiO <sub>x</sub>                | $1.44 \pm 0.01$          | $24 \pm 4$                             | (27)      |
| $\alpha$ -FeCoNiO <sub>x</sub>              | $1.42 \pm 0.01$          | $31 \pm 3$                             | (27)      |
| $\alpha$ -CoP <sub>i</sub> <sup>*</sup>     | 1.51                     | 60                                     | (28)      |
| $\alpha$ -NiB <sub>i</sub> <sup>†</sup>     | 1.53                     | 58                                     | (14)      |
| $\alpha$ -MnO <sub>x</sub>                  | 1.59                     | 76                                     | (16)      |
| LaNiO <sub>3</sub> <sup>‡</sup>             | 1.38                     | 43                                     | (7)       |
| Co <sub>3</sub> O <sub>4</sub> <sup>‡</sup> | 1.40                     | 45                                     | (29)      |
| RuO <sub>2</sub> <sup>‡</sup>               | 1.36                     | 40                                     | (9)       |
| IrO <sub>2</sub> <sup>‡</sup>               | 1.44                     | 40                                     | (30)      |

\*P<sub>i</sub> = phosphate. †B<sub>i</sub> = borate. ‡Crystalline phases.

for  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub> annealed at low temperatures; the  $\alpha$ -CoO<sub>x</sub> and  $\alpha$ -NiO<sub>x</sub> films revealed less uniform film morphologies. Energy dispersive x-ray spec-

troscopy verified the elemental composition of the films at six distinct points of each sample to be within 2 atomic % (table S2) (20). The

experimentally determined compositions of each of the three mixed-metal oxide films were in excellent agreement with the corresponding solution stoichiometries. These collective features indicate that the films consist of a homogeneous distribution of metal ions throughout the solid. Unlike what may arise with conventional metal oxide formation schemes (e.g., thermal decomposition, coprecipitation), the low-temperature PMOD process does not appear to lead to phase segregation.

Both  $\alpha$ -CoO<sub>x</sub> and  $\alpha$ -NiO<sub>x</sub> were, as expected, better OER catalysts than  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub> (Fig. 3, C to F). Cyclic voltammetry (Fig. 3C) revealed additional electron-transfer processes that occur before water oxidation for all three mixed-metal oxide films. The current spike indicating catalytic water oxidation began between 1.41 and 1.44 V ( $\eta = 0.18$  to 0.21 V) for the three materials. Moreover, Tafel slopes between 24 and 33 mV dec<sup>-1</sup> enabled these materials to reach 0.5 mA cm<sup>-2</sup> at potentials as low as 1.48 V ( $\eta = 0.25$  V). Steady-state electrochemistry measurements on the mixed-metal films highlight an improvement in kinetics of water oxidation compared to those displayed by each of the monometallic amorphous phases (Fig. 3D). Although the onsets of linearity were similar for  $\alpha$ -NiO<sub>x</sub>,  $\alpha$ -CoO<sub>x</sub>, and each of the mixed-metal oxides, the mixed-metal compositions containing Fe were characterized by a lower Tafel slope and are therefore more efficient electrocatalysts at higher current densities (table 1) (26). The stabilities of the films, which are inherently sensitive to film composition, are also reasonably high at a current density of 1 mA cm<sup>-2</sup> (e.g., a mere 6- and 30-mV increase in electrode potential was required to maintain a constant current density for  $\alpha$ -NiO<sub>x</sub> and  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub>,

respectively; fig. S9) (20). Each mixed-metal composition in this first generation of OER catalysts produced by PMOD exhibits catalytic parameters that approach those of the most active catalysts in the literature (Table 1). Given the broad applicability of this approach and the acute stoichiometric control of the metal compositions, we contend that the PMOD technique opens an entirely new parameter space for discovery and optimization of heterogeneous electrocatalysts.

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#### Supplementary Materials

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Materials and Methods  
Figs. S1 to S9  
Tables S1 and S2

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## Europe-Wide Dampening of Population Cycles in Keystone Herbivores

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Suggestions of collapse in small herbivore cycles since the 1980s have raised concerns about the loss of essential ecosystem functions. Whether such phenomena are general and result from extrinsic environmental changes or from intrinsic process stochasticity is currently unknown. Using a large compilation of time series of vole abundances, we demonstrate consistent cycle amplitude dampening associated with a reduction in winter population growth, although regulatory processes responsible for cyclicity have not been lost. The underlying syndrome of change throughout Europe and grass-eating vole species suggests a common climatic driver. Increasing intervals of low-amplitude small herbivore population fluctuations are expected in the future, and these may have cascading impacts on trophic webs across ecosystems.

**S**mall cyclic herbivores such as voles, lemmings, moths, and grouse are keystone prey for predators in many temperate and

arctic ecosystems (1, 2). There are concerns that multiple instances of population cycle collapses documented in rodents could have profound

effects on predators, alternative prey, and forage plants (3–6). Current evidence on the extent of the phenomenon mostly relies on a reasonably large but qualitative collection of anecdotes (4). The lack of a systematic and quantitative characterization of the patterns, however, makes generalization and prediction difficult. Quantitative analyses of changes in vole dynamics have invoked local explanations, such as changes in land use (7) or climate (8, 9). However, without adequate temporal and spatial coverage, correlational studies have little power to distinguish between deterministic processes caused by large-scale environmental change and transient patterns caused by random environmental perturbations. A major challenge is to understand whether the patterns of cycle collapse and their underlying processes are local or more general phenomena (10).

Prevailing models for rodent population dynamics assume that cycles are mostly determined by delayed density dependence (a reduction of population growth rate by, for example, specialist predators, plant anti-herbivore defenses, or pathogens responding to past vole density), whereas



direct density dependence (negative effect of current density through, for example, competition for resources) has a more limited contribution (11). In this setting, theory suggests three broad, nonexclusive pathways by which high-amplitude population cycles can fade out. First, in the presence of large environmental noise, quasi-cycles models commonly produce transient periods lacking visible cycles, without change in intrinsic population growth rates or density-dependent processes (pathway 1) (4). Second, sustained environmental change may affect seasonal, density-independent population growth rates, affecting cycle amplitude (pathway 2). Third, sustained environmental change may weaken interactions with biotic agents responsible for delayed density dependence (12), affecting the population's propensity to fluctuate cyclically (pathway 3) (8). We analyzed time series of vole population dynamics from a range of ecosystems across Europe in order to investigate which of these three pathways are empirically supported.

Identifying a common syndrome of change between populations would signify a shared environmental driver. We looked for such coherent changes in the time series parameters, focusing on population growth rates and direct and delayed density dependence estimated statistically by using log-linear autoregressive (AR) models. Whereas wavelet analysis (10) decomposes amplitude and frequency variation in time series, AR models decompose series into deterministic

and stochastic components, including observation error, environmental noise, intrinsic population growth rate, and density dependence. Pathways of change can therefore be directly mapped to changes in AR model parameters. We used an extension of the seasonal AR model that has been used to describe temporal variation in population regulation around a given point in time (9, 13, 14).

Most vole-monitoring schemes in Europe involve some degree of spatial replication, with up to more than 50 sampling sites per study area. In most cases, however, data have been aggregated before analysis and publishing. Spatial aggregation of time series can introduce artificial dampening and distort estimates of population growth rate and density dependence (15). We collated raw data for a large proportion of the long-term (>18 years) time series available across Europe, sampled biannually and with known sampling effort, taking advantage of the spatial replication to improve parameter estimation.

Cycles in vole communities are understood to result from two-way trophic interactions involving large-sized, mostly grass-eating vole species (*Microtus spp.*) and in some cases *Myodes rufocanus*, whereas fluctuations of competitively inferior species (such as *Myodes glareolus* and *M. rutilus*) are entrained by shared natural enemies (16–18). For our analysis, we selected the large and dominant species in each site. We left moss-eating lemmings out because they are present only in Fennoscandia and assumed processes underpinning density dependence are different (19, 20).

Our results show that out of our panel of 12 populations (Fig. 1), 10 (83%) underwent at least a twofold decline in cycle amplitude in spring, and eight populations (67%) did in both spring and autumn. In most of Europe, declines were most marked in spring, and series displayed low amplitudes around 1995 to 2005, thus generalizing patterns reported from northern Fennoscandia and the United Kingdom (9, 21). The *Microtus* populations in Eastern Germany and Poland are the only exceptions to the general pattern of cycle dampening.

Winter population growth rate trajectories declined in a way that is consistent with the declines in spring amplitude (Fig. 2A), the only exception being *Myodes rufocanus* at Pallasjärvi, North Finland (site-specific results are available in fig. S1). Summer growth rates showed trends opposite to winter (Fig. 2B) but in a way weakly related to amplitude (absolute correlation  $\leq 0.34$ ) (fig. S4). Changes in direct density dependence were inconsistent and of small magnitude, typically equivalent to a 1-year change in cycle period length (Figs. 1 and 2C).

The strength of delayed density dependence fluctuated in most populations but largely independently of amplitude (Fig. 2D). Although local changes in delayed density dependence were in some cases large enough to suggest the loss

of intrinsic cyclicity, these were neither coherent between populations nor consistent over time (Figs. 1 and 2D). Loss of delayed density dependence—and hence of cycles—was therefore not necessary for amplitude dampening to occur, which is inconsistent with pathway 3.

Previous system-specific correlative studies have tried to explain shifts between cyclic and noncyclic vole population dynamics (8–10, 12, 21). However, strong causal inference or the ruling out of purely random fluctuations (pathway 1) has been hampered by the models fitted (supplementary text VII) and lack of sufficient independent spatial or temporal replication to make general inference. If caused by intrinsic process stochasticity, a transient loss of cycles would be expected to occur independently in different systems (18). Across Europe, however, parallel declines in spring amplitude and in winter population growth rates occurred broadly at the same time for different species in farmlands, forests, and alpine and boreal habitats across a large range of latitudes with very different local climates, which is inconsistent with pathway 1.

Uncertainty about the temporal changes in parameters is large and likely to remain so for any single data series. Only through use of the type of meta-analysis we present here can we get the large-scale insight and sufficient power to isolate change in winter population growth rates (pathway 2) as a parsimonious proximal process, thus providing strong support for the hypothesis of extrinsic cyclic dampening. Our results broaden the processes contributing to the pattern of variation in vole cycles to include not only the prevailing delayed density dependence paradigm (pathway 3) but also the maximal seasonal population growth rate. This unifies the understanding of population dynamics of voles with that of insects and lemmings, for which cycle dampening has been associated with access to resources (12, 22, 23). General vole cycle dampening is therefore possible, even though the processes responsible for cycles may be local. However, pattern coherence suggests that global explanations involving large-scale environmental changes are more likely than local ones (such as snow hardness or habitat fragmentation) to explain the reduction in winter population growth rates.

Although climatic forcing is a natural candidate for explaining changes occurring at the scale of Europe and across highly different ecosystems, research is now required to identify the precise variable (or variables) affecting seasonal population growth rates in voles. It is difficult with fixed census dates to infer whether large-scale variation in seasonal growth rates relates to phenological changes (overall annual growth unchanged but shifting with respect to census dates) or changes in the mean without change in timing. Invoking phenological changes in reproduction to explain declines in winter population growth rate would imply a later average onset of reproduction, which is opposite to global

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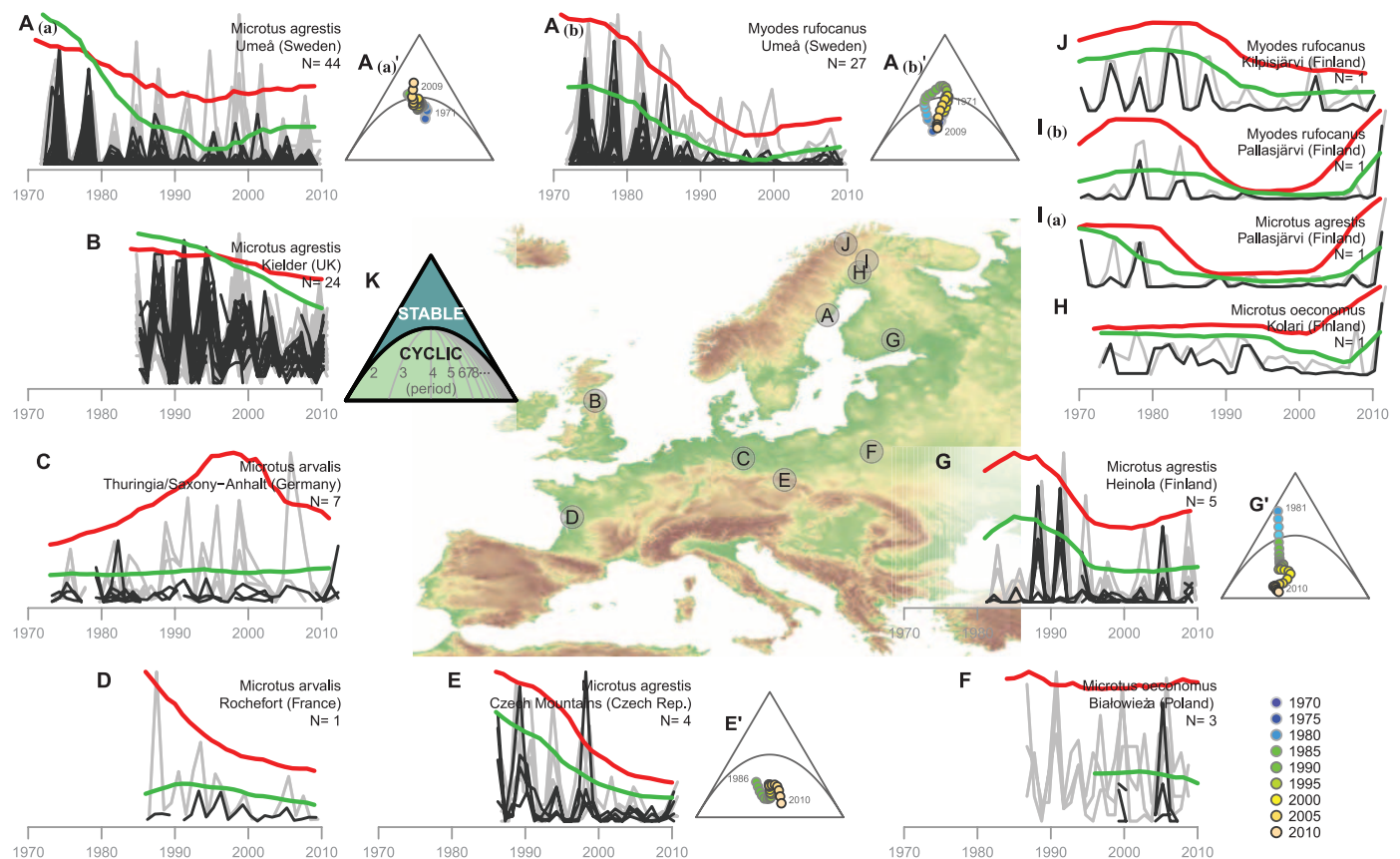
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patterns. Neither empirical evidence nor hypothetical mechanisms exist for this. Increasingly negative winter population growth rates (stronger winter declines) reduce population densities at the onset of the next breeding season, thus limiting the incremental growth of the population

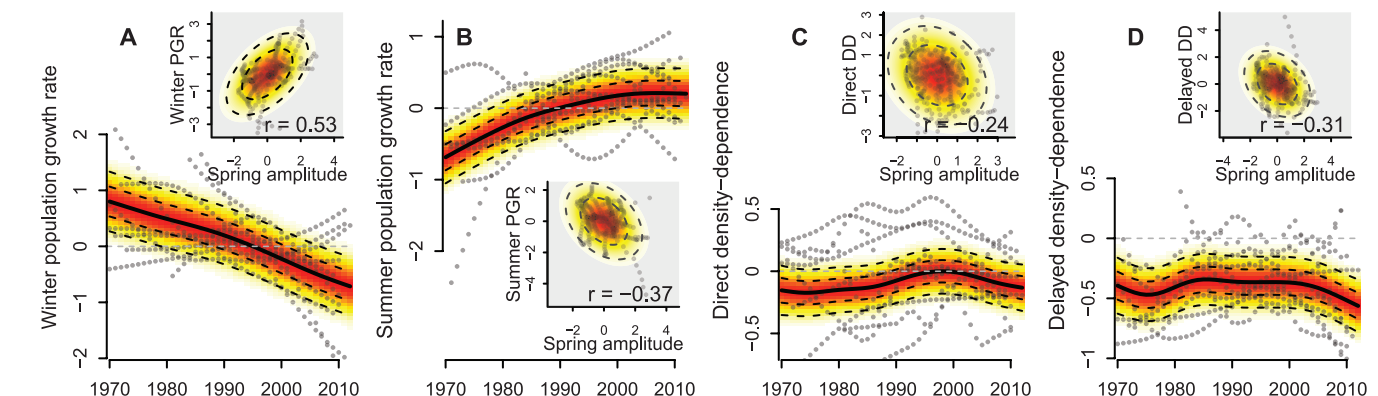
over several years (21). This may, for instance, involve a degradation of wintering conditions affecting survival (24). New theoretical research is needed to explain how contrasts in seasonal growth rates (25) affect vole population dynamics (26). Spatial gradients provide some evidence

that snow-melt patterns affected vole cycles in Fennoscandia (2); however, these would not account for cycle dampening in temperate, essentially snow-free areas such as the Atlantic West of France and northern England. Quantifying short- and long-term variation in vole



**Fig. 1. Changes in grass-eating vole population dynamics in Europe.** (A to J) Time series of vole abundance indices for spring (dark gray) and autumn (light gray). The origin of the series is shown on the central map. [Source: European Environment Agency] Green (spring) and red (autumn) curves show variation in the amplitude of fluctuations by using kernel-smoothed 90% quantiles. (K) Parameter plane of the log-linear autoregressive model (30)

(x axis, direct density dependence; y axis, delayed density dependence). The triangle defines regions producing stationary dynamics, either stable (top) or with multiannual cycles (bottom). (A<sub>(a)</sub>'), (A<sub>(b)</sub>'), (E'), and (G') illustrate examples of inconsistent temporal changes in the trajectories of the density dependence regulation parameters despite similar declines in amplitude. N indicates the number of spatial replicates of time series per data set.



**Fig. 2. Analysis of vole population dynamics parameters.** (A to D) Temporal changes in the four main parameters of the seasonal autoregressive model across populations (solid lines). (Insets) Correlation ellipses between each parameter and the amplitude of vole fluctuations, both centered and scaled by population. Color gradients represent confidence, with dashes marking the 68%

(1 SD) and 95% (2 SD) intervals for the trends and the 68 and 95% regions for the correlations (insets). Gray dots show annual estimates from each vole population (population-specific results are available in fig. S1). Only winter population growth rates (A) display a marked general change over time across populations and a strong correlation with cycle amplitude.

mortality or breeding phenology, including frequency of winter reproduction, should be part of future research, as should quantifying vegetation quality that could reflect, for example, continent-scale long-term variation in climate or nutrient deposition.

Irrespective of the proximate process (or processes) affecting winter population growth rate, the coherence of the changes coinciding with a period of ongoing global environmental change suggests increasingly frequent prolonged periods of low amplitude, although high-amplitude vole peaks—as seen in 2011 in northern Fennoscandia—may occasionally reappear. The loss of years of super-abundant voles could reduce zoonotic disease risk and crop damage (27). Continent-scale collapses in population cycles are likely to be deleterious for vole predators because for most, reproduction is modulated by vole density in spring, which is when the strongest and most consistent dampening occurs. Large impacts on vegetation (6) and predator populations (1, 28) could see cascading effects on other compartments of the food webs (3, 29) in ecosystems as diverse as farmland, forest, and arctic tundra.

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#### Supplementary Materials

www.sciencemag.org/cgi/content/full/340/6128/63/DC1  
Materials and Methods  
Supplementary Text  
Figs. S1 to S6  
BUGS Code  
Data  
References (31–47)

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## Detection and Learning of Floral Electric Fields by Bumblebees

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Insects use several senses to forage, detecting floral cues such as color, shape, pattern, and volatiles. We report a formerly unappreciated sensory modality in bumblebees (*Bombus terrestris*), detection of floral electric fields. These fields act as floral cues, which are affected by the visit of naturally charged bees. Like visual cues, floral electric fields exhibit variations in pattern and structure, which can be discriminated by bumblebees. We also show that such electric field information contributes to the complex array of floral cues that together improve a pollinator's memory of floral rewards. Because floral electric fields can change within seconds, this sensory modality may facilitate rapid and dynamic communication between flowers and their pollinators.

Flowers produce a diverse range of cues and attractants to pollinators and in doing so act as sensory billboards (1). The diversity of floral cues encompasses intricate color hues and patterns, petal texture, fragrant volatiles, local air humidity, and echolocation fingerprints (1–4). The impact of floral cues on pollinator behavior has been observed since Aristotle (5), yet new floral cues are still being discovered (3, 4). Multimodal floral cues have been found to enhance both pollinator foraging efficiency and

pollination (6), and thus facilitate increased seed and fruit set.

Flying insects, including pollinators like honeybees, usually possess a positive electric potential (7–10). Conversely, flowers often exhibit a negative potential (7, 11). Electric fields arising as a result of this potential difference between flowers and insects promote pollen transfer and adhesion over short distances (7, 8, 12, 13). Furthermore, these fields differ according to the pollination status of the flower, as the deposition of pollen and resulting pollination changes flower electric potential (14, 15). However, the use of electric fields by pollinators as informative cues has not been investigated. In the complex world of plant-pollinator interactions, any

cue that increases pollination and foraging efficiency should be mutually beneficial. Here, we report that bumblebees can detect and learn to use floral electric fields, and their structural variation, to assess floral reward and discriminate among flowers.

The electrical interactions between the bee and the flower arise from the charge carried by the bee and the potential of the flower in relation to the atmospheric electric field. To quantify bee charge, individual *B. terrestris* workers were trained to fly into a Faraday pail that contained a sucrose reward. The net charge  $q$  carried by the bee was measured from the induced voltage on a calibrated capacitor (methodology described in supplementary materials). Measured on 51 individuals, 94% of bees were positively charged and 6% negatively charged ( $q_{\text{mean}} = 32 \pm 5$  pC, SD = 35pC) (Fig. 1A). These results corroborate previous measurements on the honeybee *Apis mellifera* (9) and establish that the majority of bees flying in the arena carry a positive charge susceptible to transfer.

Electrical interaction between bee and flower was further explored by placing *Petunia integrifolia* flowers in an arena with free-flying foraging bees. The electric potential in *Petunia* stems was recorded to assess the electrical signature produced by the approach and landing of an individual charged bee. Charge transfer to the flower resulted in a positive change in electric potential recorded in the stem. The landing of 50 indi-

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viduals resulted in a mean potential change lasting ~100 s, which peaked at  $-25 \pm 3$  mV (SD = 24,  $n = 50$ ) (Fig. 1B). Such change exceeds natural fluctuations in the absence of bees (Fig. 1B) and outlasts the presence of the bee on the flower. This change in potential is often initiated before contact with the bee (movie S1), suggesting that this is not simply a hydraulic wound-response variation potential as in (16) but involves direct electrostatic induction between the charged bee and the grounded flower as hypothesized in (7, 8).

Because the floral electric potential is directly affected by pollination (14, 15) and bee visitation (Fig. 1B), it potentially carries information for other visiting pollinators regarding floral resources. Visiting pollinators affect floral cues directly, by leaving scent marks on the petal surface, or by initiating changes in floral cues, such as color, shape, and humidity (4, 17–19). Such changes typically occur in the time frame of minutes to hours. The variation potential produced by bee visitation occurs within a time frame of seconds (Fig. 1B).

For a floral electric field to act as a cue, it must be possible for pollinators to detect and

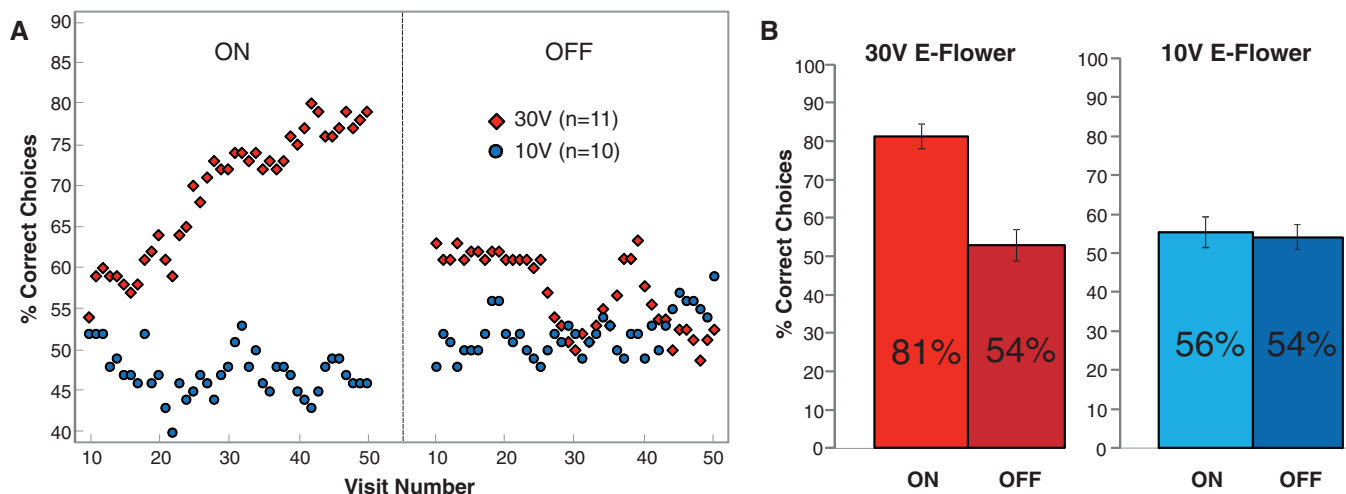
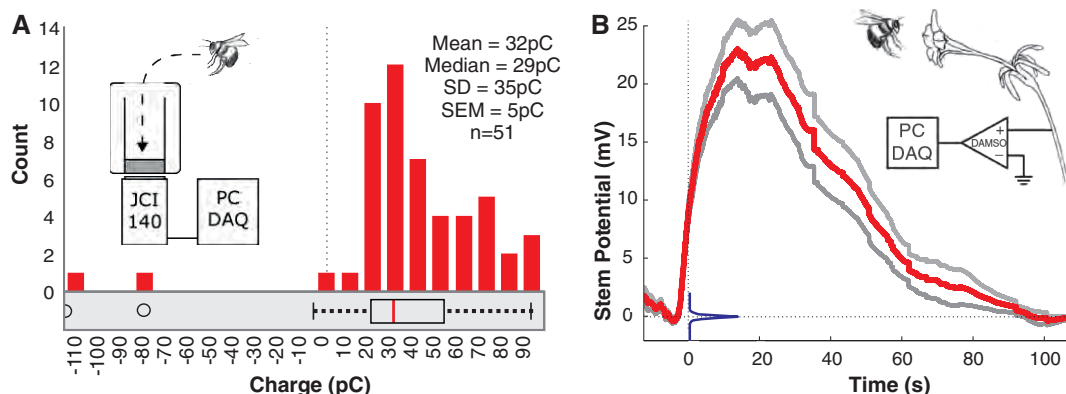
discriminate it from the background. We used differential conditioning (3) to test the ability of bumblebees to discriminate between artificial flowers (E-flowers) with differing electric fields. E-flowers consisted of a 35-mm-diameter by 1.5-mm-thick steel base disk decorated with a purple epoxy top disk. Half the E-flowers were held at a biologically relevant 30-V dc bias voltage. This voltage was chosen as a proxy for the electric field of an isolated flower standing 30-cm tall in a typical  $100 \text{ V m}^{-1}$  atmospheric electric field (20). Charged E-flowers offered a sucrose reward, while identical E-flowers were held at ground (0 V) and provided a bitter quinine hemisulfate solution (3). E-flowers were indistinguishable in every other respect. During the course of 50 bee visits, there was an increase in the relative number of visits to rewarding charged flowers (Fig. 2A). To measure bee learning, we compared the mean accuracy of the final 10 visits (visit 41 to 50) to a random choice model. In their final 10 visits to 30-V charged E-flowers, bees ( $n = 11$ ) achieved  $81 \pm 3\%$  accuracy ( $T_{1\text{-sample}} = 10.8$ ,  $P = 7.4 \times 10^{-7}$ ). Both flower types were then grounded and the choice test continued. Without the electric cue, the same

set of trained bees could no longer discriminate between the rewarding and unrewarding E-flowers, also demonstrating the absence of systematic experimental bias. Accuracy after the electric cue is removed was  $54 \pm 4\%$ , which does not differ significantly from random choice ( $T_{1\text{-sample}} = 1$ ,  $P = 0.35$ ) (Fig. 1B). Using a 10-V bias failed to elicit significant learning ( $n = 10$ , mean accuracy =  $56 \pm 4\%$ ,  $T_{1\text{-sample}} = 1.4$ ,  $P = 0.19$ ) (Fig. 2, A and B).

Floral cues are diverse and address the multimodal perception of pollinators. Working in concert, floral cues enhance foraging efficiency (6) and constitute a complex informational ecology of competing flower advertisement. Color cues rely both on hue and on contrast between hues and their geometrical patterns. Nectar guides constitute such patterns, providing information attractive to pollinators and facilitating foraging efforts (21, 22). By analogy, the geometry of floral electric fields may carry additional information important for pollinators. The diversity of floral electric field geometry can be experimentally visualized by coating flowers with positively charged colored particles released as an aerosol close to the corolla. The heterogeneous

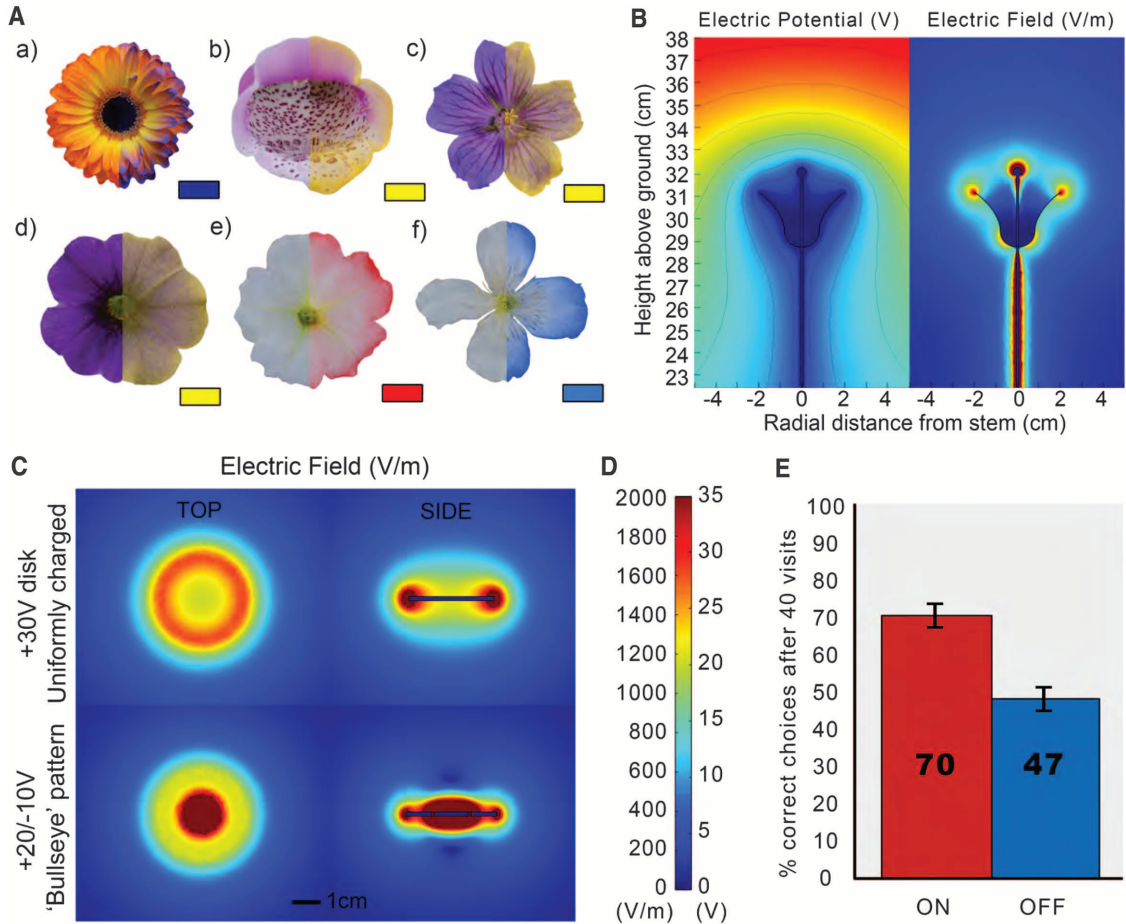
**Fig. 1. Electric charge carried by bumblebees and its transfer to flowers.**

(A) Histogram of electric charge of flying bumblebees. Boxplot shows median, SD, interquartile range, and outliers. (B) Mean variation potential in the *Petunia* stem resulting from bee landings (red,  $n = 51$ ), shown with  $\pm 1$  SEM (gray). Distribution of the natural variation of stem potential (measured along 35 samples of 30 s) in absence of bees, truncated at 2 SD (blue).

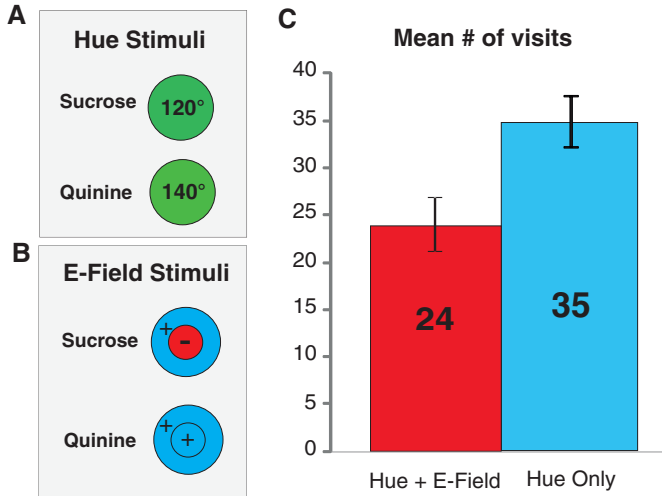


**Fig. 2. Bumblebees learn the presence of an electric field.** (A) Learning curves of foraging bees, trained to 30-V (red diamonds) or 10-V (blue circles) E-flowers. Dashed line shows switching off electric field. (B) Mean correct choices to 30-V (left) and 10-V (right) E-flowers over visits 41 to 50 in (A) during training (voltage on) and control (voltage off). Error bars show SEM.

**Fig. 3. Geometry of floral electric field and discrimination task.** (A) Flowers before (left half) and after (right) spraying with electrostatic colored powder; (a) *Gerbera hybrida*, (b) *Digitalis purpurea*, (c) *Geranium magnificum*, (d) *Calibrachoa hybrida*, (e) *Petunia hybrida*, (f) *Clematis armandii*. Density of powder deposition reflects the variation in electric field strength at the flowers' surface. (B) FE model of an idealized 30-cm-tall flower, equipotential with ground, in an atmospheric field of 100 V/m. Left: scalar electric potential. Right: electric field strength at the flowers' surface. (C) FE models of electric field produced by E-flowers. (D) Color scale for (B) and (C). (E) Pattern discrimination as mean percentage of correct choices over the last 10 visits for patterns on and off. Error bars show SEM.



**Fig. 4. Multimodal facilitation.** Colors (A) and voltage configurations (B) associated with rewarding and aversive E-flowers. (C) Mean number of visits taken by bees in each group to reach 80% correct choices.



pattern of color deposition reveals the structure of the electric field at the flower's surface (Fig. 3A). Electric field structure was also visualized using finite element (FE) modeling of an idealized 30-cm-tall flower in a physically realistic, 100 V m<sup>-1</sup> atmospheric electric field (20) (Fig. 3B, left). Plants are conductively linked to ground via their stems and roots, a connection that maintains them close to ground poten-

tial (7). Hence, a grounded 30-cm-tall plant in such an atmospheric electric field exhibits a 30-V potential difference between its inflorescent structures and the surrounding air, exhibiting a patterned electric field (Fig. 3B). This experimental and modeling evidence reveals that flower morphology determines electric field geometry. To test the bee's ability to discriminate E-field geometries, differential conditioning was used

with two types of E-flowers, providing similar voltage but different local patterns (Fig. 3C). Rewarding E-flowers presented a bull's eye pattern, with the outer ring held at +20 V and the center ring at -10 V. Aversive E-flowers presented a homogeneous voltage at +20 V (Fig. 3C). Bees ( $n = 10$ ) learned to discriminate between these two patterns, reaching  $70 \pm 3\%$  accuracy over their final 10 visits, performing significantly better than random choice ( $T_{1\text{-sample}} = 6.7$ ,  $P = 8 \times 10^{-5}$ ) (Fig. 3E). After this task, a subset of the bees ( $n = 4$ ) was allowed to complete 50 additional visits to rewarding and aversive E-flowers with identical homogeneous +20 V fields. These bees failed to discriminate between E-flowers (Fig. 3E). Altogether, these tests show that bumblebees can discriminate charged from uncharged flowers and can distinguish between flowers that differ in the geometry of their electric field. As such, E-fields could be used by flowers to provide information to their pollinators. Floral cues can work individually or complementarily (1, 6). When presented together, multimodal cues enhance the certainty of sensory information used by honeybees. Specifically, the association of color with olfactory floral cues reduces the bees' perceptual uncertainty related to an individual floral cue and increases their ability to distinguish between rewarded

and aversive stimuli (23). The hypothesis can be formulated that the floral electric field reinforces the effectiveness of other floral cues. If true, an electric cue paired with a color cue should produce an enhanced learning outcome equivalent to that obtained with the test using color and scent. Differential conditioning was used to test this hypothesis. The same two green target hues were used as in (23), but olfactory cues were replaced with a patterned electric field (Fig. 3C). Bees were trained to discriminate between E-flowers of hue 120° HSB (hue, saturation, brightness) which offered a sucrose reward, and E-flowers of hue 140° HSB, which provided an aversive quinine solution (Fig. 4A). Bees learned to discriminate between the rewarding and aversive chargeless E-flowers either using color information alone ( $n = 16$ ) or in combination with the patterned E-field ( $n = 18$ ) (Fig. 4A). When learning color on its own, discrimination to 80% success (i.e., 8 out of the last 10 choices correct) took  $35 \pm 3$  visits. When combined with the E-field pattern, the number of visits required was significantly reduced to  $24 \pm 3$  ( $T_{2\text{-sample}}; \text{unequal} = 2.86, P = 0.008$ ) (Fig. 4A). This demonstrates that the combination of two cues, E-field and hue, enhances the bee's ability to discriminate.

Our results show that electric field constitutes a floral cue. Contributing to a varied floral display aimed at pollinator senses, electric fields act to improve both speed and accuracy with which bees learn and discriminate rewarding resources. As such, electric field sensing constitutes a potentially important sensory modality, which should be considered alongside vision

and olfaction. The ubiquity of electric fields in nature and their integration into the bees' sensory ecology suggest that E-fields play a thus far unappreciated role in plant-insect interactions. The present study raises the possibility of reciprocal information transfer between plants and pollinators at time scales of milliseconds to seconds, much faster than previously described alterations in floral scent, color, or humidity (4, 18, 19). The remarkably accurate discrimination and learning of color patterns by bees was revealed by both laboratory and field training experiments (19, 21–23). Similarly, the present laboratory study reveals that floral electric fields occur in patterns and that they can be perceived. Hence, our study provides a framework for exploring the function and adaptive value of the perception of weak electric fields by bees in nature.

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#### Supplementary Materials

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Materials and Methods

References (24–26)

Movie S1

Data File S1

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## Recovery of an Isolated Coral Reef System Following Severe Disturbance

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Andrew H. Baird,<sup>2</sup> Morgan S. Pratchett<sup>2</sup>

Coral reef recovery from major disturbance is hypothesized to depend on the arrival of propagules from nearby undisturbed reefs. Therefore, reefs isolated by distance or current patterns are thought to be highly vulnerable to catastrophic disturbance. We found that on an isolated reef system in north Western Australia, coral cover increased from 9% to 44% within 12 years of a coral bleaching event, despite a 94% reduction in larval supply for 6 years after the bleaching. The initial increase in coral cover was the result of high rates of growth and survival of remnant colonies, followed by a rapid increase in juvenile recruitment as colonies matured. We show that isolated reefs can recover from major disturbance, and that the benefits of their isolation from chronic anthropogenic pressures can outweigh the costs of limited connectivity.

Coral reefs are dynamic ecosystems periodically subjected to severe disturbances, such as cyclones, from which they typically recover at scales of one to two decades (1, 2). Today, this recovery is undermined by increasing anthropogenic pressures leading to global declines in coral cover (3, 4) and diversity (5, 6). Understanding the global degradation of coral reef ecosystems requires long-term data

on population and community dynamics, especially demographic processes (7–9). However, the rarity of such data has precluded a thorough assessment of the future of coral reef ecosystems in the IPCC report on climate change (10, 11), and current knowledge is mostly derived from studies of reef degradation (9, 12) rather than reef recovery. Here, we document the recovery of coral assemblages at Australia's largest oceanic reef

system, where changes in assemblage structure and key demographic parameters were quantified for 16 years, through a regime of disturbances beginning with a catastrophic mass bleaching event in 1998.

The Scott system of reefs is surrounded by oceanic waters on the edge of Western Australia's continental shelf. It is more than 250 km from the mainland and other reefs in the region, and more than 1000 km from a major center of urbanization (fig. S1). There is little fishing pressure at the reefs, apart from the harvesting of sea cucumber, trochus, and shark fin by Indonesian islanders using traditional fishing methods for more than 300 years (13, 14). Such oceanic reef systems may provide a critical refuge for coral reef assemblages because they are far removed from most direct anthropogenic pressures. Conversely, isolation and a consequent lack of connectivity may make such systems

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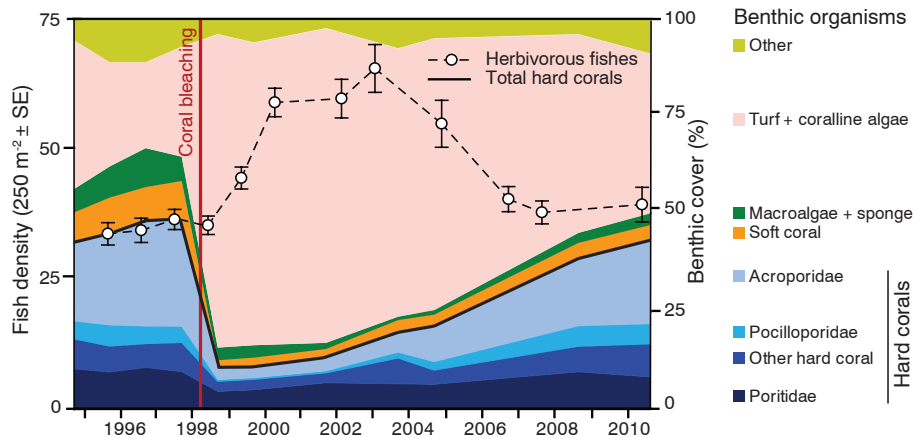
more susceptible to disturbances. Without an external supply of recruits, it is assumed that reefs will be very slow to recover from severe disturbance (15–18). However, the sensitivity of recovery to variation in larval supply and post-recruitment processes has not been investigated for a coral reef system through a full cycle of impact and recovery.

Extreme water temperatures leading to mass coral bleaching occurred in all regions of the world in 1998 (19), and the Scott Reef system was severely affected. Seawater temperatures at Scott Reef rose rapidly during February 1998 and remained above average for the next 2 months (fig. S2). The NOAA satellite estimate of the cumulative degree heating weeks, a measure of the severity of the temperature anomaly, was 13.3°C; this remains the most extreme temperature anomaly recorded at Scott Reef (20). Catastrophic mortality of corals across the entire reef system occurred over the next 6 months

to depths of 20 m. Along permanent transects at replicate sites across Scott Reef (13), between 80 and 90% of live coral was lost from the reef crest (~3 m) and reef slope (~9 m), and almost 70% from the upper reef slope (~6 m) (fig. S3A). On the reef slope, the coral assemblage changed markedly and the number of genera decreased by half (Fig. 1 and fig. S3). The reductions in coral cover were followed by recruitment failure to settlement tiles (21), which were redeployed ( $n = 108$ ) at the permanent transects on the reef slope (13). In the years before the bleaching, the total number of recruits was between 2600 and 5600; this had decreased to zero a year later, clearly indicating that larvae were locally derived. For 6 years, recruitment rates were <6% of those prior to the disturbance (Fig. 2), and initial increases in coral cover were driven by the growth of remnant corals. On the basis of these rates of change, recovery was projected to take decades. Within

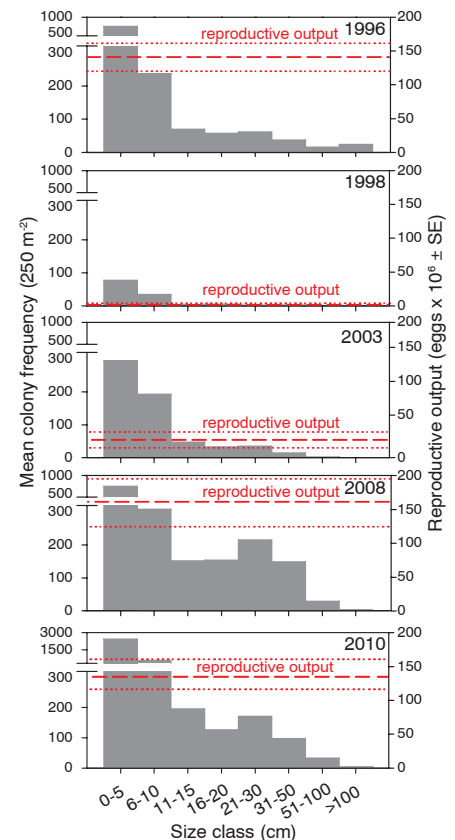
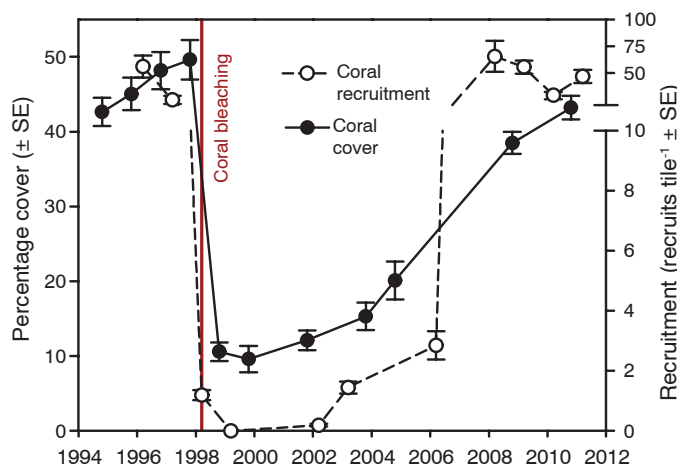
12 years, however, coral cover, recruitment, generic diversity, and community structure were again similar to the prebleaching years (Figs. 1 and 2 and fig. S3).

The decline in recruitment after the mass mortality of corals in 1998 was caused by the drastic reduction in local brood stock (Fig. 3) and a negligible supply of larvae from neighboring reefs hundreds of kilometers away (22, 23). However, no phase shift to macroalgae was observed; the substrata made available by the death of corals was colonized by fine turfing and coralline algae (Fig. 1) and not by macroalgae, sponges, or other organisms that can exclude and outcompete corals (24, 25). The already high densities of herbivorous fishes also increased after the loss of coral (Fig. 1), probably in response to the changes in turfing algae (26); this finding suggests a surplus grazing capacity within the system that assisted subsequent coral recruitment and survival (27). Consequently, a high proportion of the coral larvae were produced locally, settled, and survived. The



**Fig. 1. Temporal dynamics in benthic communities on the reef slope at Scott Reef.** Marked declines in the cover of corals (especially Acroporidae) followed the mass coral bleaching in March 1998. There was a corresponding increase in the cover of fine turfing and coralline algae, but the cover of sponges, macroalgae, and other benthic organisms remained low. The mean ( $\pm$ SE) density of herbivorous fishes increased for several years after the mass bleaching event. Coral assemblages had mostly recovered by 2010, despite two cyclones (2004 and 2007), an outbreak of disease (2009), and a moderate bleaching event (2010).

**Fig. 2. Stock-recruitment relationship for corals on the reef slope at Scott Reef.** The mass bleaching in 1998 caused an 80% decline in coral cover (mean percent  $\pm$  SE) and a 94% decline in recruitment (mean number per tile  $\pm$  SE) of the dominant coral taxa, the genus *Acropora*, over 6 years (note axis break for recruit density; recruitment was not measured in 2007). A decade after the mass bleaching, cover and recruitment were similar to or higher than before the disturbance.



**Fig. 3. Temporal dynamics in size structure and reproductive output of *Acropora* on the reef slope at Scott Reef.** The mean reproductive output (solid red line) and SE (dashed red lines) were calculated from the assemblage size structure and size-specific fecundity of the most common species, *A. spicifera*. In 2008, a decade after the mass bleaching, the reproductive output was similar to before the disturbance, coinciding with the rapid increase in coral recruitment (Fig. 2).

mean survival of recruits (<5 cm;  $n = 1281$ ) of branching (*Acropora*) and massive (*Goniastrea*) corals during the recovery period (13) ranged from 83 to 93% each year (fig. S4), which is far higher than the <50% survival of recruits on reefs experiencing chronic pressures (28, 29). Indeed, the mean survival of all ( $n = 5333$ ) colonies was consistently higher than 80% each year, apart from the lower survival (>53% year<sup>-1</sup>) of some larger (>15 cm) branching corals at sites exposed to cyclonic waves in 2007 (fig. S4). High survival and growth resulted in rapid rates of transition through increasing colony size classes, with corresponding increases in brood stock and reproductive output (13) (Fig. 3). Reproductive output and recruitment were similar to pre-disturbance levels within a decade of the bleaching, and 2 years later, coral cover and community structure had also recovered.

The recovery of corals at Scott Reef after the 1998 mass bleaching may have been even faster if not for a series of more moderate disturbances, including two cyclones, an outbreak of disease, and a second bleaching. This demonstrates that even coral reefs with a negligible supply of larvae from outside can recover relatively quickly from disturbances in the absence of chronic human pressures. Other ecosystems have displayed a similar resilience when environmental conditions were not fundamentally altered by human activities (30). Our results suggest that addressing local pressures, such as pollution and overfishing, is as important to the recovery of coral reefs as the establishment of networks of marine protected areas (MPAs). Managing local pressures to promote resilience will be critical

to preventing the global degradation of coral reefs, with climate change likely to cause additional severe disturbances in the near future.

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#### Supplementary Materials

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Materials and Methods

Figs. S1 to S4

Table S1

References (31–40)

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## Mechanism-Based Covalent Neuraminidase Inhibitors with Broad-Spectrum Influenza Antiviral Activity

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Influenza antiviral agents play important roles in modulating disease severity and in controlling pandemics while vaccines are prepared, but the development of resistance to agents like the commonly used neuraminidase inhibitor oseltamivir may limit their future utility. We report here on a new class of specific, mechanism-based anti-influenza drugs that function through the formation of a stabilized covalent intermediate in the influenza neuraminidase enzyme, and we confirm this mode of action with structural and mechanistic studies. These compounds function in cell-based assays and in animal models, with efficacies comparable to that of the neuraminidase inhibitor zanamivir and with broad-spectrum activity against drug-resistant strains *in vitro*. The similarity of their structure to that of the natural substrate and their mechanism-based design make these attractive antiviral candidates.

The envelope of the influenza virus contains two immunodominant glycoproteins, hemagglutinin (HA) and neuraminidase

(NA), that play key roles in viral infection and spread. HA effects attachment of the virus to the host cell through its interaction with surface sialic

acids, thereby initiating entry. Once the virus has replicated, the NA cleaves sialic acids from the viral and cell surfaces, allowing the virus progeny to spread to uninfected cells. On the basis of the notion that potent and specific viral NA inhibitors should function to reduce viral spread, structure-based inhibitor design programs have

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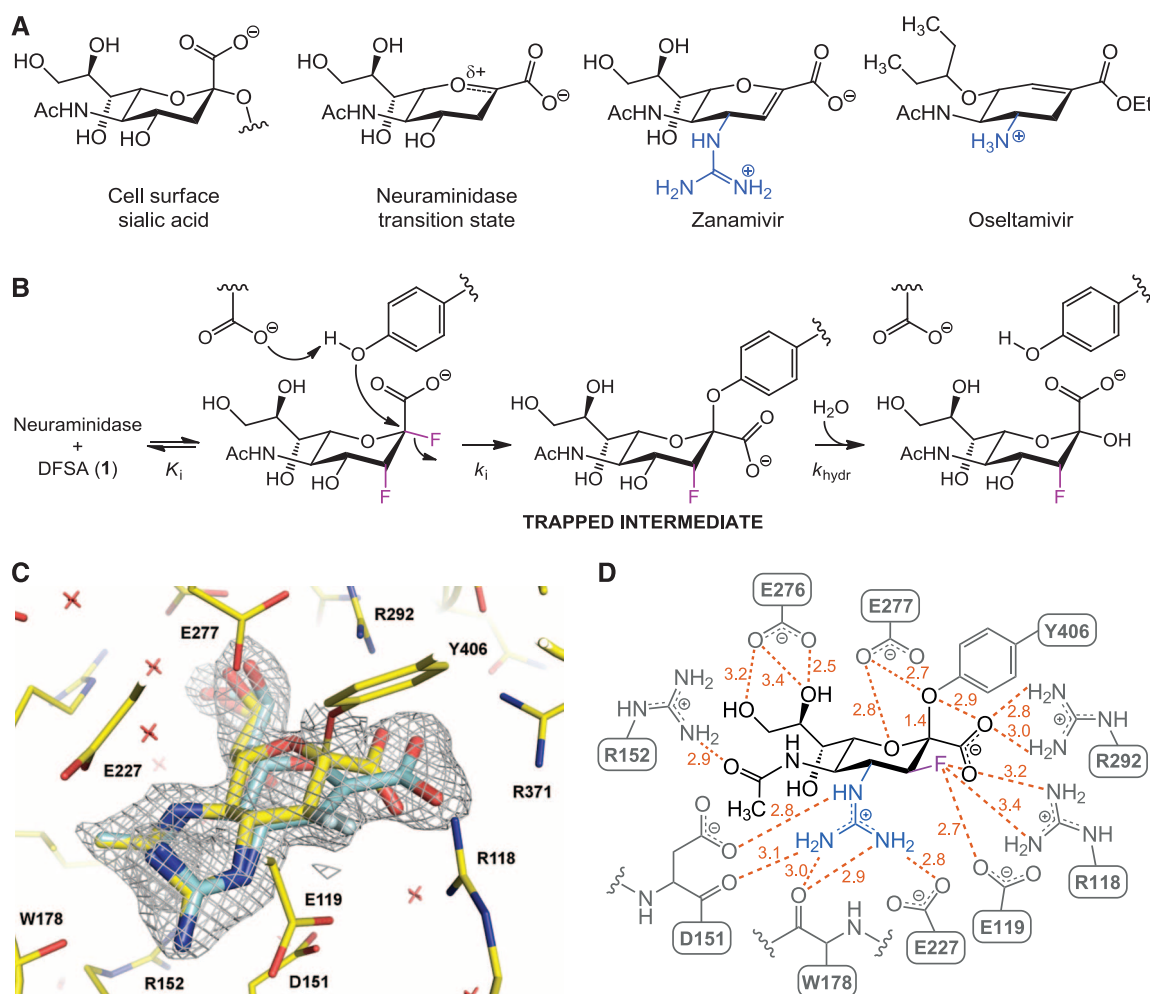
produced two widely used anti-influenza drugs, zanamivir (Relenza) and oseltamivir (Tamiflu) (1) (Fig. 1A). These reversible inhibitors were designed to mimic the transition state, with a guanidinium or ammonium substituent added at the position corresponding to C-4 of the natural substrate to bind in an anionic pocket, thereby increasing affinity and specificity over human NAs. However, drug-resistant strains are now emerging, particularly against the more widely used and structurally divergent drug oseltamivir, highlighting an urgent need for new classes of NA inhibitors that differ minimally in structure from the parent sialic acid, given that the development of resistance to structurally conservative, mechanism-based inhibitors should be a much less probable event (2–10).

NAs catalyze the hydrolysis of sialosides with net retention of stereochemistry at the site of substitution. A mechanism involving an ion-pair intermediate has long been suggested for the GH34

(11) influenza NA (12), although involvement of a covalent intermediate, as has been shown for GH33 NAs (13), has emerged as an alternative. We provide evidence for such a covalent intermediate by use of 2,3-difluorosialic acid (**1**, DFSA) (Fig. 1B) as a substrate that exhibits slow turnover. The electronegative fluorine atom at C-3 inductively destabilizes the oxocarbenium ion-like transition states for both formation and hydrolysis of the intermediate, thus slowing each step, whereas the C-2 anomeric fluoride leaving group speeds the formation step, permitting accumulation of the covalent intermediate (Fig. 1B). Rapid inactivation of N9 NA was observed at low inactivator concentrations, such that individual kinetic parameters ( $K_i$  and  $k_i$ ) could not be determined; only a second-order rate constant  $k_i/K_i$  of  $196 \text{ min}^{-1} \text{ mM}^{-1}$  could be measured. Turnover of the covalent intermediate ( $k_{\text{hydr}}$ ) also occurred rapidly, with a  $t_{1/2} < 1 \text{ min}$ . Confirmation of the formation of a covalent species and identification

of the site of attachment was achieved by peptic digestion of N9 NA that had been labeled with DFSA 1, or by its difluoroKDN analog (figs. S1 to S3). Isolation and subsequent sequence analysis of the labeled peptide by liquid chromatography-based tandem mass spectrometry identified this peptide as NTDWSGYSSSGSF, with the tyrosine (Y) bearing the sugar label, thereby confirming that Y406 functions as a catalytic nucleophile.

Knowing that the influenza NAs employ a covalent mechanism, we explored these DFSAs as a possible new class of covalent mechanism-based influenza therapeutics. These are attractive because the initial affinity of the drug ( $K_i$ ) can be optimized, as well as the relative rate constants for formation ( $k_i$ ) and hydrolysis ( $k_{\text{hydr}}$ ) of the trapped intermediate, with the objective being to optimize the ratio of  $k_i/k_{\text{hydr}}$ . Versions of **1** bearing amine (Am) and guanidine (Gu) substituents at C-4 were of interest because these cationic substituents might improve the initial affinity and

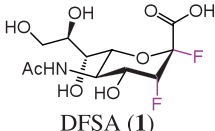
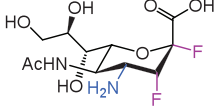
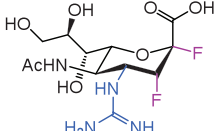
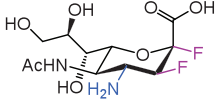
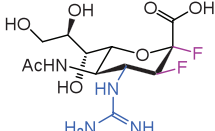


**Fig. 1. Structures of key influenza therapeutics, mechanism of action of DFSAs, and x-ray structure of inhibited enzyme.** (A) Chemical structures of cell surface sialic acids, the neuraminidase transition state, zanamivir (Relenza), and oseltamivir (Tamiflu). (B) Mechanism of action of the DFSAs. (C) X-ray crystallographic structure of the active site of the enzyme trapped as its 3-fluoro(eq)-4-quanidino-sialyl-enzyme intermediate (elimination product is in pale cyan) overlaid

with omit (22) electron density map shown as a gray mesh contoured at  $1\sigma$  within 1.6 Å of ligands. The electron density extends from the ligand molecule to Y406, suggesting a covalent link between the inhibitor's C-2 atom and the OH of Y406. **(D)** Diagram of interactions (orange dashed lines; distances in Å) with the sialic acid in the covalently inhibited enzyme. The corresponding diagram of interactions for the elimination product is shown in fig. S4.



**Table 1. Inactivation and reactivation parameters for difluorosialic acids (DFSAs).\***

| Compound                                                                                              | Virus <sup>†</sup>  | $k_i/K_i$<br>( $\text{min}^{-1}\text{mM}^{-1}$ ) | $t_{1/2}$ (inac)<br>(min) | $t_{1/2}$ (reac)<br>(min) |
|-------------------------------------------------------------------------------------------------------|---------------------|--------------------------------------------------|---------------------------|---------------------------|
| <br>DFSA (1)         | G70C H1N9           | 196                                              | N.D. <sup>‡</sup>         | < 1                       |
| <br>FaxAmDFSAs (2)   | Brisbane H1N1       | 106                                              | 2.1                       | 6900                      |
|                                                                                                       | Brisbane H1N1 H275Y | 29                                               | 2.5                       | 3450                      |
|                                                                                                       | California H1N1     | 95                                               | 1.7                       | 6900                      |
|                                                                                                       | G70C H1N9           | 74                                               | 4.0                       | 2300                      |
|                                                                                                       | Hong Kong H3N2      | 140                                              | 20.8                      | >6900                     |
| <br>FaxGuDFSAs (3)   | Brisbane H1N1       | 371                                              | 7.4                       | >6900                     |
|                                                                                                       | Brisbane H1N1 H275Y | 160                                              | 9.2                       | >6900                     |
|                                                                                                       | California H1N1     | 93                                               | 20.9                      | >6900                     |
|                                                                                                       | G70C H1N9           | 246                                              | 6.8                       | >6900                     |
|                                                                                                       | Hong Kong H3N2      | 470                                              | 20.2                      | >6900                     |
| <br>FeqAmDFSAs (4)  | Brisbane H1N1       | 3479                                             | 0.9                       | 256                       |
|                                                                                                       | Brisbane H1N1 H275Y | 849                                              | 0.2                       | 117                       |
|                                                                                                       | California H1N1     | 4422                                             | 0.4                       | 46                        |
|                                                                                                       | G70C H1N9           | 4332                                             | 0.8                       | 49                        |
|                                                                                                       | Hong Kong H3N2      | 5662                                             | ---                       | 153                       |
| <br>FeqGuDFSAs (5) | Brisbane H1N1       | 5812                                             | 0.3                       | 1380                      |
|                                                                                                       | Brisbane H1N1 H275Y | 2992                                             | 0.5                       | 460                       |
|                                                                                                       | California H1N1     | 7594                                             | 0.9                       | 690                       |
|                                                                                                       | G70C H1N9           | 3879                                             | 0.5                       | 363                       |
|                                                                                                       | Hong Kong H3N2      | 5737                                             | 0.8                       | 1380                      |

\*Refer to table S4 for full kinetic parameters. <sup>†</sup>Brisbane H1N1, A/Brisbane/59/07; Brisbane H1N1 H275Y, A/Brisbane/59/07 oseltamivir-resistant; California H1N1, A/California/07/09; G70C H1N9, A/NWS/G70C/75; Hong Kong H3N2, A/HongKong/01/68. <sup>‡</sup>N.D., Not determined.

**Table 2. IC<sub>50</sub> values (nM) in the enzyme inhibition assay for wild-type and mutant pairs. Boldface indicates resistance to zanamivir or oseltamivir. IC<sub>50</sub> is the concentration of inhibitor that reduces enzyme activity by 50% compared to the control uninhibited value. Values are the means of duplicate assays.**

| Virus*                   | Zanamivir    | Oseltamivir  | DFSAs (1) | FaxAm<br>DFSAs (2) | FaxGu<br>DFSAs (3) | FeqAm<br>DFSAs (4) | FeqGu<br>DFSAs (5) |
|--------------------------|--------------|--------------|-----------|--------------------|--------------------|--------------------|--------------------|
| B/Perth                  | 8.9          | 104.4        | 70        | 210                | 54                 | 5.4                | 4.5                |
| B/Perth D197E            | <b>257.5</b> | <b>708.0</b> | 170       | 340                | 162                | 16                 | 8                  |
| A/Mississippi H1N1       | 1.9          | 3.1          | 80        | 840                | 115                | 45                 | 13                 |
| A/Mississippi H1N1 H275Y | 2.2          | <b>2440</b>  | 120       | 2210               | 217                | 86                 | 44                 |
| A/Fukui H3N2             | 3.8          | 1.7          | 1380      | 4710               | 2006               | 71                 | 25                 |
| A/Fukui H3N2 E119V       | 3.4          | <b>260.0</b> | 240       | 2440               | 998                | 265                | 2.4                |
| G70C H1N9                | 2.7          | 2.8          | 1190      | 2700               | 66.7               | 270                | 140                |
| G70C H1N9 E119G          | <b>678.4</b> | 2.9          | 1150      | 1600               | 1433               | 73                 | 17                 |

\*B/Perth, B/Perth/211/01; B/Perth D197E, B/Perth/211/01 zanamivir and oseltamivir-resistant; A/Mississippi H1N1, A/Mississippi/3/01; A/Mississippi H1N1 H275Y, A/Mississippi/3/01 oseltamivir-resistant; A/Fukui H3N2, A/Fukui/45/01; A/Fukui H3N2 E119V, A/Fukui/45/01 oseltamivir-resistant; G70C H1N9, A/NWS/G70C/75; G70C H1N9 E119G, A/NWS/G70C/75 zanamivir-resistant

the specificity for the influenza enzyme and also slow down the turnover of the intermediate, both through improved interactions of the intermediate species with the enzyme and by inductively destabilizing the reaction transition state. The effects of axial (ax) or equatorial (eq) stereochemistry of F3 on inhibitory behavior were also explored. Synthesis of the protected diastereomeric 3-axial and 3-equatorial-2,3-difluoro-4-azido neuraminic acids as key intermediates was achieved by Selectfluor hydroxyfluorination of 2,4-dideoxy-2,3-didehydro-4-azido-N-acetylneuraminic acid (4-azido-DANA) (14) followed by installation of an equatorial fluorine at C-2 using diethylaminosulfur trifluoride (DAST). The lead candidates shown in Table 1—FaxAmDFSAs (2), FaxGuDFSAs (3), FeqAmDFSAs (4), and FeqGuDFSAs (5)—were then prepared by reduction or reductive guanidylolation, followed by deprotection (15).

Kinetic parameters for inactivation and reactivation of N1, N2, and N9 NAs, as representative influenza group 1 and group 2 enzymes, by 2 to 5 are presented in Table 1 and table S4, along with parameters for the parent DFSAs with the N9 NA. Not only did incorporation of the charged substituent at C-4 result in high initial affinity, but also, more importantly, it greatly reduced the rate constant for reactivation of enzymes inactivated by 2, 3, 4 and 5, with half-lives for reactivation now ranging from 0.75 hours to >100 hours. The viral NA will therefore remain inactivated for extended times, even after the compound may have been cleared from relevant tissues, with favorable consequences for pharmacokinetic behavior. Interestingly, compounds with an equatorial fluorine at C-3 inactivated and reactivated faster than did those with an axial fluorine, typically by a factor of 10 to 40. Furthermore, a guanidine substituent at C-4 slowed both the inactivation and reactivation more than did an amine substituent, with a much greater effect on the reactivation step. This difference in rates likely has its origins in optimized interactions of the guanidine with the active site at the stage of the covalent intermediate, as is seen in the crystal structure of the trapped species shown in Fig. 1C. A covalent bond of 1.45 Å is observed in the electron density map (Fig. 1C and table S1), between C-2 of 3-fluoro (eq) sialic acid and the phenolic oxygen of Y406, and the C-4 guanidine indeed forms strong interactions with the anionic pocket, very similar to those found with zanamivir (1). As observed previously in a structure of the GH33 sialidase NanI (16), the covalent intermediate species is accompanied by an unsaturated form of fluorosialic acid formed by elimination.

An absolute comparison of the in vitro efficacy of these compounds as enzyme inhibitors with those of zanamivir or oseltamivir is difficult given their different modes of action, covalent versus noncovalent, and the time dependence of inhibition (17). However, a pragmatic measure was achieved by measuring median inhibitory concentration (IC<sub>50</sub>) values for each DFSAs, as well as zanamivir and oseltamivir carboxylate,

against four different virus strains and resistant mutants after preincubation for 30 min before substrate addition (Table 2). In almost all cases, each compound with an equatorial fluorine was a superior inhibitor to its axial epimer. Likewise, in each case the guanidine derivative showed superior performance to its amine analog. Consequently, compound **5** (FeqGuDFSA) was the optimal derivative, with IC<sub>50</sub> values comparable to those for zanamivir and oseltamivir, “on rates” that are generally superior to those of zanamivir, and off rates that are lower (1, 18, 19).

The specificity of these inhibitors was then evaluated by testing them against Neu2 as a representative human NA (all human NAs belong to the sequence-related family GH33). No inactivation of Neu2 was seen with either of the amine derivatives. Inactivation by FaxGuDFSA (**3**) and FeqGuDFSA (**5**) occurred slowly, but at rates lower by a factor of 10<sup>5</sup> to 10<sup>6</sup> than those seen for inactivation of influenza NA at comparable concentrations. By comparison, zanamivir inhibits Neu2 with a K<sub>i</sub> of 17 μM, a factor of only 10<sup>3</sup> to 10<sup>4</sup> higher than for influenza NA (20).

On the basis of these results, the ability of compounds **2** to **5** to inhibit the replication of the virus in cell culture was explored using MDCK cells (Madin-Darby canine kidney cells). Three

A strains (N1, N2, and N9) plus one B strain were tested in plaque size reduction assays (PRA), with zanamivir as control. All DFSAs inhibited virus replication (Table 3 and figs. S5 to S8) with no cytotoxicity observed, even at 5 mM DFSA concentrations, as measured by neutral red staining of viable cells. For all strains, substitution with the 4-amino group enhanced inhibition of virus replication over the parent DFSA, and the 4-guanidino substitution was better still. Likewise the presence of an equatorial rather than axial fluorine in the 4-guanidino version resulted in a further 10-fold enhancement in potency for three of the strains tested. Consequently, FeqGuDFSA (**5**) had the highest potency of all of the inhibitors, including zanamivir, against the influenza B virus (fig. S5) and performed comparably against the influenza A strains. Importantly, the PRA data largely mirror the in vitro kinetic data, with the compounds possessing an equatorial fluorine being superior to those with an axial fluorine, and the 4-guanidino substitution being superior to a 4-amino. Furthermore, the absolute IC<sub>50</sub> values in these PRAs were consistently lower than those for enzyme inhibition for all compounds except FeqAmDFSA (**4**) and in all cases were in the low nanomolar range. The DFSA derivatives also proved effective in vitro against NAs from a series

of resistant strains, with FeqGuDFSA (**5**) again proving to be the most widely active (Table 2). All compounds proved effective against the oseltamivir-resistant H275Y mutant, wherein binding of the isopentyl side chain of oseltamivir is compromised. Importantly, the E119G mutation, which disrupts interactions with the 4-guanidine of zanamivir (reduction in efficacy by a factor of 250) (**5**) only reduced the efficacy of FaxGuDFSA (**3**) by a factor of 20. Notably, the efficacy of FeqGuDFSA (**5**) was enhanced by a factor of ~10 for the E119G- and oseltamivir-resistant E119V mutants. Selection against transition state analog binding (zanamivir) clearly does not equivalently suppress covalent intermediate accumulation. This excellent profile against otherwise resistant strains supports the concept of mechanism-based inhibition as a means to minimize selection of resistant strains.

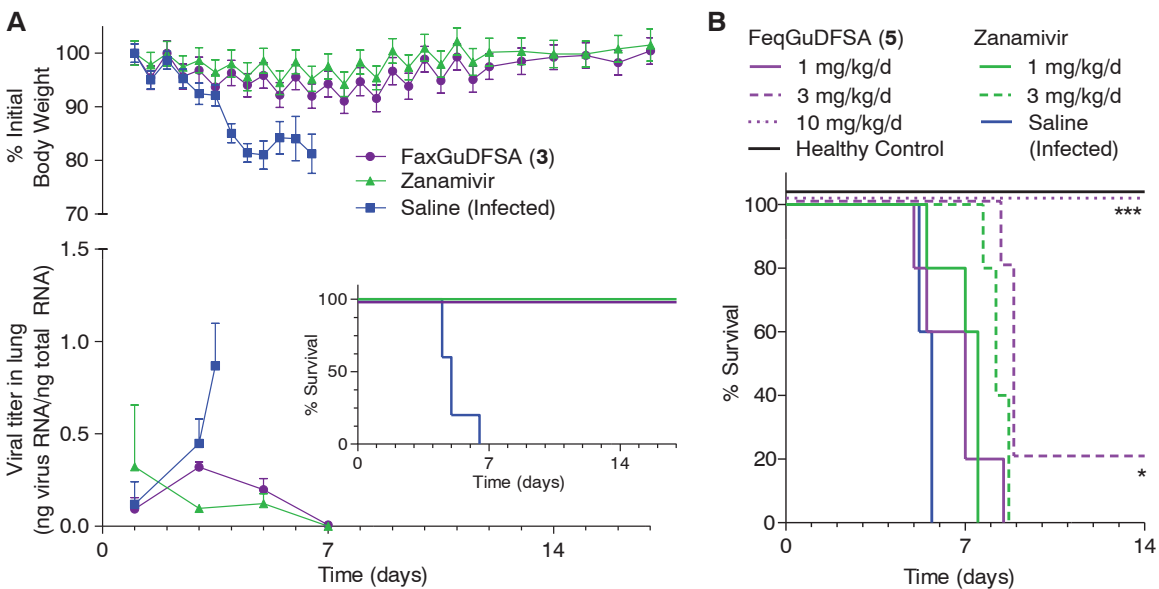
Before commencing in vivo efficacy studies in mouse models, the pharmacokinetic properties of FaxGuDFSA (**3**) as a representative DFSA derivative were evaluated for administration by intravenous and intranasal routes and shown to be comparable to those collected in parallel for zanamivir, as shown in table S2. Efficacy tests were then conducted using a mouse-adapted influenza A virus strain, A/Hong Kong/1/68 (H3N2) (21). Balb/c mice were treated with either DFSA derivative, zanamivir, or saline twice daily by intranasal administration, starting 2 hours before infection. At a dose of 1 mg per kg of weight per day (mg/kg/day), FaxGuDFSA showed superior results to FaxAmDFSA in prolonging survival. At a higher dose of 10 mg/kg/day, it protected all the mice from the lethal infection, as did zanamivir (Fig. 2A). Measurement of viral RNA loads in lung tissue confirmed that survival was indeed associated with suppression of viral replication.

**Table 3. IC<sub>50</sub> values in the plaque size reduction assay.** Values are the means of duplicate assays.

| Virus*             | Zanamivir | DFSA (1) | FaxAm DFSA (2) | FaxGu DFSA (3) | FeqAm DFSA (4) | FeqGu DFSA (5) |
|--------------------|-----------|----------|----------------|----------------|----------------|----------------|
| B/Perth            | 10 nM     | 1 μM     | 100 nM         | 10–100 nM      | 10–100 nM      | 1 nM           |
| A/Mississippi H1N1 | ≤1 nM     | 1 μM     | 100 nM         | 10 nM          | 100 nM         | 1 nM           |
| A/Fukui H3N2       | 100 nM    | 100 μM   | 1 μM           | 100 nM         | 1 μM           | 10 nM          |
| G70C H1N9          | 1–10 nM   | 1–10 μM  | 1 μM           | 1–10 nM        | 1 μM           | 10 nM          |

\*B/Perth, B/Perth/211/01; A/Mississippi H1N1, A/Mississippi/3/01; A/Fukui H3N2, A/Fukui/45/01; G70C H1N9, A/NWS/ G70C/75.

**Fig. 2. Efficacy of FaxGuDFSA (**3**), FeqGuDFSA (**5**), and zanamivir in treating H3N2 influenza infection in the Balb/c mouse. (A) The top graph shows body weight over the 17-day observation period. Animals that lost 20% of initial weight were recorded as non-survivors, as indicated in the survival plot (inset). Ten animals per group were used for this experiment. In another set of animals, viral RNA loads were measured over 7 days by quantitative real-time fluorescence polymerase chain reaction (bottom graph). The entire lung tissue was collected from four animals at each time point for each treatment except for the last time point for the saline control. In this experiment, all saline control animals survived less than 4 days, and the last time point for this group**



shown is at 80 hours after infection (seven animals). (B) Dose-dependent efficacy of FeqGuDFSA (**5**) (1 to 10 mg/kg/day) and zanamivir. \*Mantel Cox  $P = 0.03$ ; \*\*\*Mantel Cox  $P < 0.001$ . Five animals per group were challenged and treated as indicated.

Likewise, dose dependency of FeqGuDFSA in the protection of mice was demonstrated with 100% efficacy at 10 mg/kg/day (Fig. 2B and table S3), with no ill effects in the treated animals during the experiments compared with the saline control.

The DFSAs represent a distinct class of potent, mechanism-based, specific NA inhibitor functioning by transient formation of a covalent intermediate species. Their high efficacy as enzyme inhibitors is matched by excellent antiviral activity in cell-based plaque size reduction assays, at levels similar or superior to those for zanamivir. Furthermore, they show good inhibition of NAs from zanamivir- or oseltamivir-resistant influenza virus strains, indicating an altered resistance profile. Most important, they function well in controlling influenza infections in an animal model, at levels comparable to those used for zanamivir.

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# Decameric SelA•tRNA<sup>Sec</sup> Ring Structure Reveals Mechanism of Bacterial Selenocysteine Formation

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experiments (table S1) were performed with *Escherichia coli* Sela (*A. aeolicus* numbering is used in this Report, fig. S1).

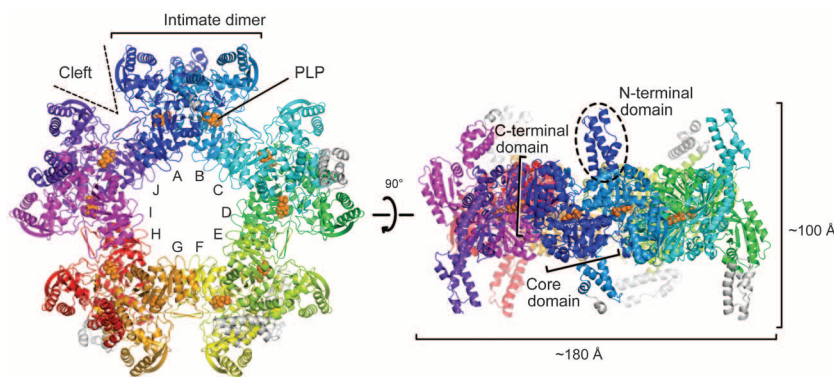
Sela is a homodecamer in which the 10 subunits form a pentamer of dimers (Fig. 1). Each subunit consists of the N-terminal domain (residues 1 to 66), the N-linker (residues 67 to 89), the core domain (residues 90 to 338), and the C-terminal domain (residues 339 to 452) (fig. S2A). The intimate dimer in Sela contains two catalytic sites formed at the subunit-subunit interface, where the cofactor PLP is covalently linked to a conserved Lys<sup>285</sup> (fig. S2, B to E). The N-terminal domain protrudes from the central pentagon, is intrinsically mobile (fig. S3),

and only contacts the other core domain of the intimate dimer. The orientation of the C-terminal domain (relative to the core domain) differs substantially from that of the C-terminal domains in other fold-type-I PLP-dependent enzymes (7) (fig. S4). Its orientation creates a large space between the core and C-terminal domains (fig. S2A), which allows the interaction with the neighboring intimate dimer for decamerization (fig. S4B) and the formation of a large cleft between two intimate dimers (Fig. 1).

The structure (Fig. 2A) of *A. aeolicus* Sela complexed to *T. tengcongensis* tRNA<sup>Sec</sup> at 7.5-Å resolution (cocrytals with the homologous tRNA only diffracted to ~20 Å) revealed that the Sela

decamer binds up to 10 tRNA<sup>Sec</sup> molecules. Despite the low resolution of the ~0.8-MD ribonucleoprotein, the positions of the tRNA<sup>Sec</sup> molecules were unambiguously detected in the electron density map, except for the CCA terminus (Fig. 2B). Four Sela subunits interact with one Ser-tRNA<sup>Sec</sup>, with one dimer holding tRNA<sup>Sec</sup> and the other providing the catalytic site (Fig. 2B). The large cleft (Fig. 1) provides the space for tRNA<sup>Sec</sup> to approach the catalytic site on the neighboring dimer. Therefore, the proper relative positioning of the two dimers is required to perform the overall reaction and is fixed by the ring closure to form the decameric structure (Fig. 2C). If Sela assumed the quaternary structure of any other fold-type-I PLP enzyme [e.g., the tetrameric SepSecS (8)], the tRNA-binding and catalytic sites could not work together. In fact, a quadruple mutation introduced at the dimer-dimer interface (Thr<sup>191</sup>-Thr<sup>192</sup>-Asp<sup>199</sup>-Tyr<sup>220</sup>→Tyr<sup>191</sup>-Tyr<sup>192</sup>-Arg<sup>199</sup>-Pro<sup>220</sup>) caused a dimeric quaternary structure (fig. S5A) and abolished Sela activity in vivo (table S1) and in vitro (fig. S5B).

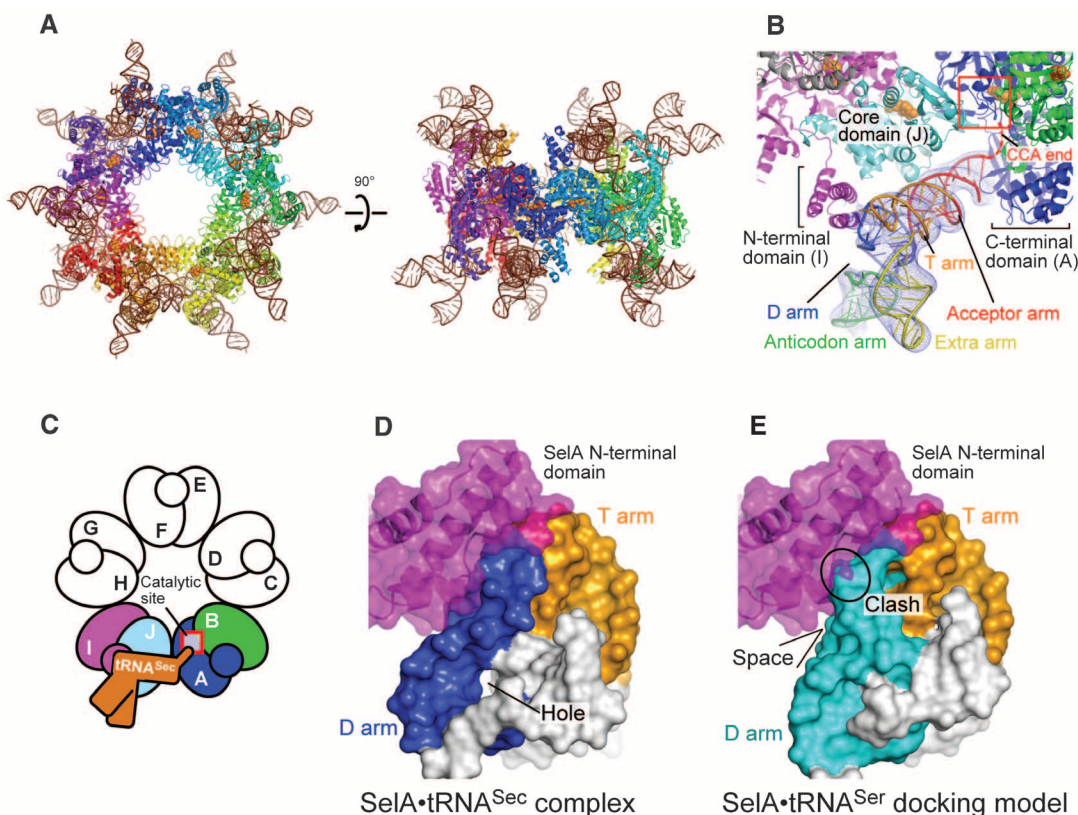
Sela interacts with the D-arm side of the L-shaped tRNA<sup>Sec</sup> and does not contact either the extra arm or the anticodon arm (Fig. 2B). The N-terminal domain of Sela binds the D arm and the T loop of tRNA<sup>Sec</sup> (fig. S6, A and B). The Sela-ΔN mutant, lacking the N-terminal domain, did not bind Ser-tRNA<sup>Sec</sup> or tRNA<sup>Sec</sup>, whereas the dimeric mutant dSela, which retains the N-terminal domain, can still bind tRNA<sup>Sec</sup> (fig. S5, C and D). The dissociation constant



**Fig. 1. Structure of Sela.** Overall structure of *A. aeolicus* Sela. PLPs are represented as sphere models. The 10 subunits are shown in different colors. The disordered regions in the N-terminal domains are colored gray.

## Fig. 2. Structure of the Sela•tRNA<sup>Sec</sup> complex.

(A) Overall structure of *A. aeolicus* Sela complexed with *T. tengcongensis* tRNA<sup>Sec</sup> (colored brown). (B) Interaction of tRNA<sup>Sec</sup> with intimate dimers |J| and A•B (close-up view). The omit  $F_{\text{obs}} - F_{\text{calc}}$  map (3.0 sigma level) of the tRNA<sup>Sec</sup> is shown in a blue mesh. The putative position of the CCA region is shown as a dashed line. (C) A schematic diagram of the interaction of tRNA<sup>Sec</sup> with dimers |J| and A•B. (D and E) Discrimination of tRNA<sup>Sec</sup> from tRNA<sup>Ser</sup> by Sela. *T. thermophilus* tRNA<sup>Ser</sup> [PDB ID 1SER (14)] was docked to the Sela•tRNA<sup>Sec</sup> complex by superimposing the backbone phosphorus atoms of the T arm. The tRNA<sup>Sec</sup> surface is complementary to that of the Sela N-terminal domain (D), whereas that of tRNA<sup>Ser</sup> is not complementary (E).





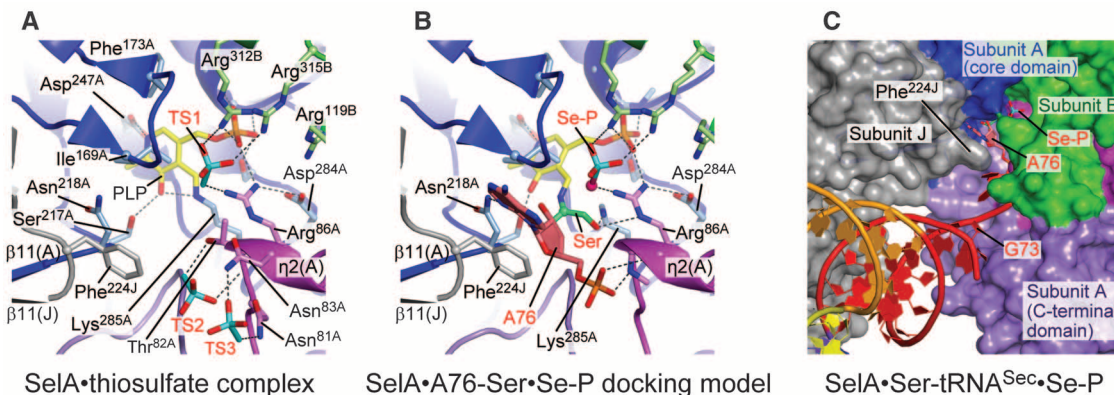
( $K_D$ ) for dSelA is  $624 \pm 84$  (SD) nM, whereas that for the wild type is  $75 \pm 13$  nM, indicating that more than 90% of the standard change in Gibbs free energy ( $\Delta G^\circ$ ) for tRNA<sup>Sec</sup> binding is ascribed to the tRNA<sup>Sec</sup> interactions within one dimer, particularly with the N-terminal domain. The specific interaction between the SelA N-terminal domain and the tRNA<sup>Sec</sup> D arm accomplishes the discrimination of the substrate Ser-tRNA<sup>Sec</sup> from the structurally divergent nonsubstrate Ser-

tRNA<sup>Ser</sup> [the maximal six-base-pair D stem and minimal four-nucleotide D loop in tRNA<sup>Sec</sup> compared with the three-base-pair D stem and 11-nucleotide D loop in tRNA<sup>Ser</sup> (fig. S7)]. The D stem of *T. tengcongensis* tRNA<sup>Sec</sup> contains the fifth and sixth pairs G14:U21 [G14:C21 in *A. aeolicus* tRNA<sup>Sec</sup> (fig. S7)] and C15:G20a, whereas D loop nucleotide U16 forms the unique tertiary base pair U16:U59. The SelA N-terminal domain contacts C15, U16, G20a, and U21 be-

cause the shape of the unique D-arm structure of tRNA<sup>Sec</sup> is complementary to the protein structure (Fig. 2D and fig. S6D). Because of the incompatible shape of tRNA<sup>Ser</sup>, the corresponding nucleotides in the canonical augmented D helix of tRNA<sup>Ser</sup> cannot interact with the SelA N-terminal domain (Fig. 2E and fig. S6D). In contrast, the difference in the number of base pairs in the acceptor stem between tRNA<sup>Sec</sup> and tRNA<sup>Ser</sup> (eight and seven, respectively)

### Fig. 3. Catalytic site of SelA.

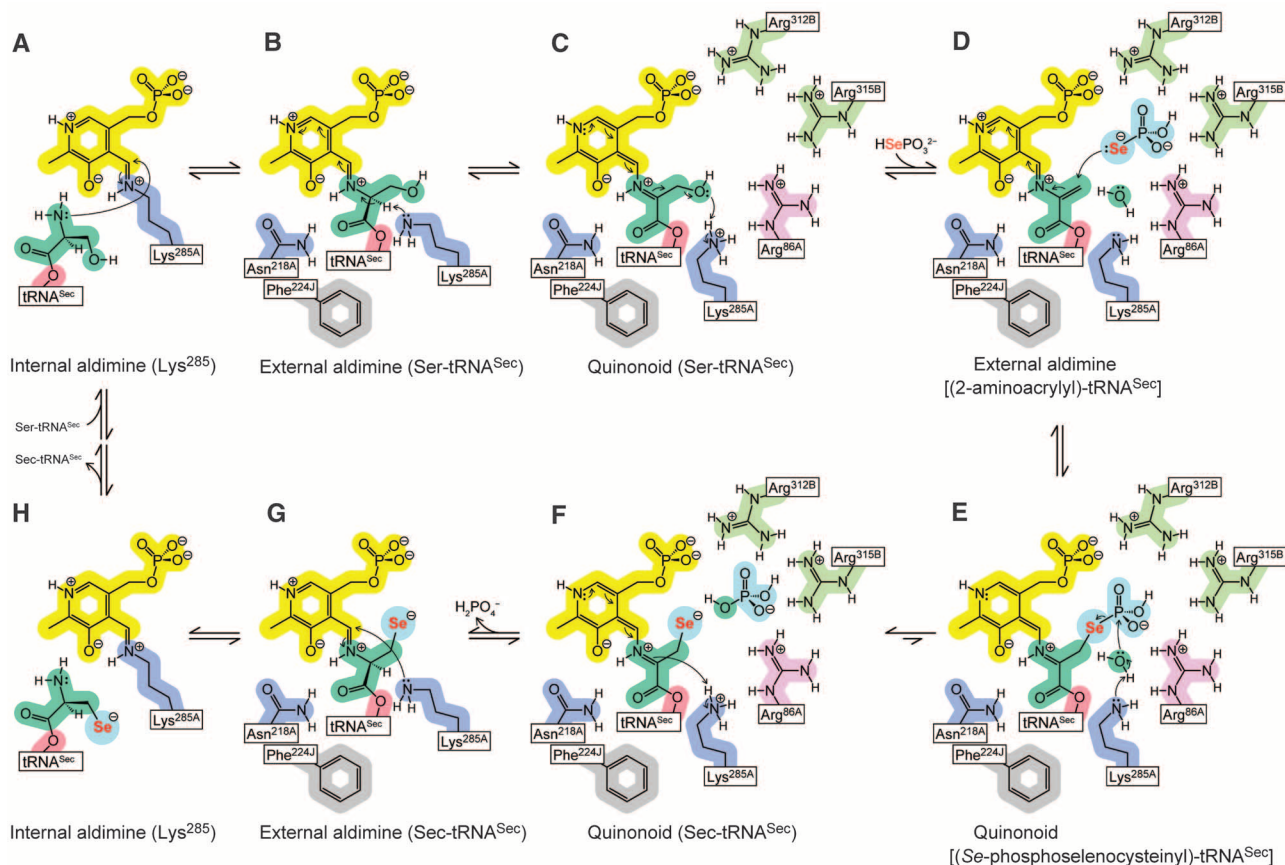
(A) The catalytic site of SelA- $\Delta$ N in complex with thiosulfate, PLP and thiosulfate ions (TS1 to TS3) are shown. TS1 mimics the substrate selenophosphate (Se-P). The subunits of the intimate dimer A\*B are colored as in fig. S2A, whereas subunit J is colored gray. (B) Docking model of the Ser-ligated terminal tRNA<sup>Sec</sup> nucleotide (A76) and Se-P. (C) SelA surface, showing that A76 binds to the boundary of subunits J and A. The docked A76-Ser and G73 of tRNA<sup>Sec</sup> are connected through C74-C75.



SelA•thiosulfate complex

SelA•A76-Ser•Se-P docking model

SelA•Ser-tRNA<sup>Sec</sup>•Se-P



**Fig. 4. The mechanism of Sep-to-Sec conversion.** In the waiting state, the PLP forms an internal aldimine with Lys<sup>285</sup> (A). The amino group of Ser-tRNA<sup>Sec</sup> replaces Lys<sup>285</sup> to form an external aldimine (B), followed by deprotonation of the  $\alpha$  position of the Ser moiety (C). Lys<sup>285</sup> protonates and

eliminates the  $\beta$  hydroxyl group to form a 2-aminoacrylyl moiety (D). The nucleophilic addition of selenophosphate generates a Se-phosphorylated Sec moiety (E), which is hydrolyzed to a Sec moiety and a phosphate (F). Lastly, the  $\alpha$  position of the Sec moiety is protonated (G), and Sec-tRNA<sup>Sec</sup> is released (H).



barely contributes to the discrimination, probably because of the inherent flexibility of the N-terminal domain (fig. S6D).

The large cleft described above enables SelA to hold the end of the acceptor stem: G1 from the G1:C72 pair and the G73 discriminator nucleotide interact with the C-terminal domain of the neighboring subunit (Fig. 2B and fig. S6C). The deletion of residues 423 and 424 within the  $\beta$ 18-loop region, which contacts G73 (fig. S6C), yielded completely inactive enzymes (table S1), indicating that the binding of G1 and G73 is required for properly directing the acceptor arm toward the catalytic site. Although the CCA end is not visible, probably because tRNA<sup>Sec</sup> is not ligated with serine, the length of CCA-Ser is consistent with the distance between G73 in tRNA<sup>Sec</sup> and PLP in the catalytic site (fig. S6C). Therefore, the large cleft can accommodate the terminal region, from the first base pair of the acceptor stem to the Ser-adenosine moiety of Ser-tRNA<sup>Sec</sup> (fig. S8A), whereas the N-terminal domain recognizes the tRNA<sup>Sec</sup>-specific D-arm structure. In contrast, SepSecS binds Sep-tRNA<sup>Sec</sup> on the surface of the protein globule in a completely different manner from that of SelA and is therefore unable to recognize the D arm (fig. S8, B and C).

We also determined the crystal structure of SelA- $\Delta$ N in complex with thiosulfate, which revealed thiosulfate ions (TS1 to TS3) bound to one SelA subunit (Fig. 3A, fig. S9A). TS1 binds in the putative selenophosphate-binding pocket formed by Arg<sup>86A</sup> (subunit A), Arg<sup>312B</sup>, and Arg<sup>315B</sup> (subunit B). Mutations of these residues to Ala resulted in markedly reduced activities. Furthermore, Arg<sup>119</sup> and Asp<sup>284</sup>, which interact with Arg<sup>312B</sup> and Asp<sup>86A</sup>, respectively, are required for catalytic activity (table S1). TS2 and TS3 are thought to mimic the phosphate group of the terminal A76 of tRNA<sup>Sec</sup>. Because the Ala mutations of Asn<sup>218</sup> and Phe<sup>224</sup> drastically reduced the activity in vivo (table S1), Asn<sup>218A</sup> and Phe<sup>224J</sup> might form part of the A76-binding pocket at the interface between the subunits A and J (Fig. 3, B and C). Notably, the interaction of A76 with both Asn<sup>218A</sup> and Phe<sup>224J</sup> can only occur in the decameric SelA (fig. S9B). The mechanism of PLP-dependent Ser-to-Sec conversion by SelA (depicted in Fig. 4 and explained in the legend) is similar to that of SepSecS (8) and, unlike other fold-type-I PLP enzymes, involves a series of crucial Arg residues. However, SelA and SepSecS use respective sets of nonhomologous Arg residues.

In summary, the SelA pentamer-of-dimers forms a ring-shaped structure and binds 10 tRNA<sup>Sec</sup> molecules. Each tRNA<sup>Sec</sup> interacts with all subunits from the two neighboring dimers, and this interaction critically depends on the pentagonal ring architecture. The SelA N-terminal domain recognizes the tRNA<sup>Sec</sup>-specific D-arm structure, thereby discriminating Ser-tRNA<sup>Sec</sup> from Ser-tRNA<sup>Ser</sup>. Moreover, the large cleft created between two SelA dimers accommodates the Ser-tRNA<sup>Sec</sup> 3'-terminal region. Thus, the func-

tional form of SelA differs from that of the tetrameric SepSecS, which recognizes the phosphoserine moiety of Sep-tRNA<sup>Sec</sup> without tRNA<sup>Sec</sup>/tRNA<sup>Ser</sup> discrimination. Moreover, SelA catalyzes Sec formation by using a different set of active-site Arg residues from that of SepSecS. The entirely different structures and divergent catalytic residues of the bacterial and archaeal/eukaryotic Sec synthases are evidence for convergent evolution of two Sec synthesis systems present in nature.

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## Supplementary Materials

www.sciencemag.org/cgi/content/full/340/6128/75/DC1  
Materials and Methods  
Figs. S1 to S9  
Tables S1 and S2  
References (15–28)

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# Drosophila H1 Regulates the Genetic Activity of Heterochromatin by Recruitment of Su(var)3-9

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Eukaryotic genomes harbor transposable elements and other repetitive sequences that must be silenced. Small RNA interference pathways play a major role in their repression. Here, we reveal another mechanism for silencing these sequences in *Drosophila*. Depleting the linker histone H1 in vivo leads to strong activation of these elements. H1-mediated silencing occurs in combination with the heterochromatin-specific histone H3 lysine 9 methyltransferase Su(var)3-9. H1 physically interacts with Su(var)3-9 and recruits it to chromatin in vitro, which promotes H3 methylation. We propose that H1 plays a key role in silencing by tethering Su(var)3-9 to heterochromatin. The tethering function of H1 adds to its established role as a regulator of chromatin compaction and accessibility.

Eukaryotic genomes are packaged into chromatin, which is composed of highly conserved repetitive units referred to as nucleosomes. The nucleosome consists of ~145 base pairs of DNA wrapped around an octamer of core histones, H2A, H2B, H3, and H4. Chromatin also contains the linker histone H1, which binds to the linker DNA between nucleosomes and facilitates folding of nucleosome arrays into more compact structures (1). Chromatin is organized into regions of euchromatin and more densely packed heterochromatin, which is generally

silenced. The mechanisms leading to heterochromatic silencing are not well understood (2, 3).

Depleting H1 in *Drosophila* by RNA interference (RNAi) leads to marked disruption of salivary gland (SG) polytene chromosome structure, including pericentric heterochromatin, and a decrease in nucleosome spacing (4). We compared the RNA expression profiles of SGs depleted of H1 and control *Nautilus* (Nau) RNAi SGs. We found only a modest difference in the mRNA profile, with only 2174 (11.5%) protein-coding genes showing a change of twofold or more ( $P <$

0.05) (Fig. 1A). However, H1 depletion caused significant changes in the abundance of transcripts derived from transposable elements (TEs). Of the 79 annotated TE transcripts, the abundance of 42 (53.2%) changed by twofold or more (Fig. 1A and table S1), with more than 98% of changes representing increased expression. Quantitative reverse transcription polymerase chain reaction (QRT-PCR) of 11 different RNAs representing various classes of *Drosophila* TEs confirmed that their expression is activated from 15-fold to as much as 800-fold (Fig. 1B). Thus, the repressive function of H1 in vivo is directed particularly toward TEs.

Preferential repression of TEs is also observed in normal, mitotically dividing *Drosophila* cells. RNA expression profiling of Kc cells depleted of H1 to ~30% of control levels (fig. S1A) showed similar effects on derepression of TE transcripts and significant overlap with those observed in SGs, as well as a similar limited effect on protein-coding genes (fig. S1B). Furthermore, measurements of transposon transcript levels by QRT-PCR in four other tissue sources (whole larvae, brains, ovaries, and testes) from H1-depleted animals showed that expression of TEs is activated from 50- to more than 500-fold (fig. S2, A and B). Therefore, H1 exerts a strong repressive effect on TE expression in a variety of cell types [see also (5)].

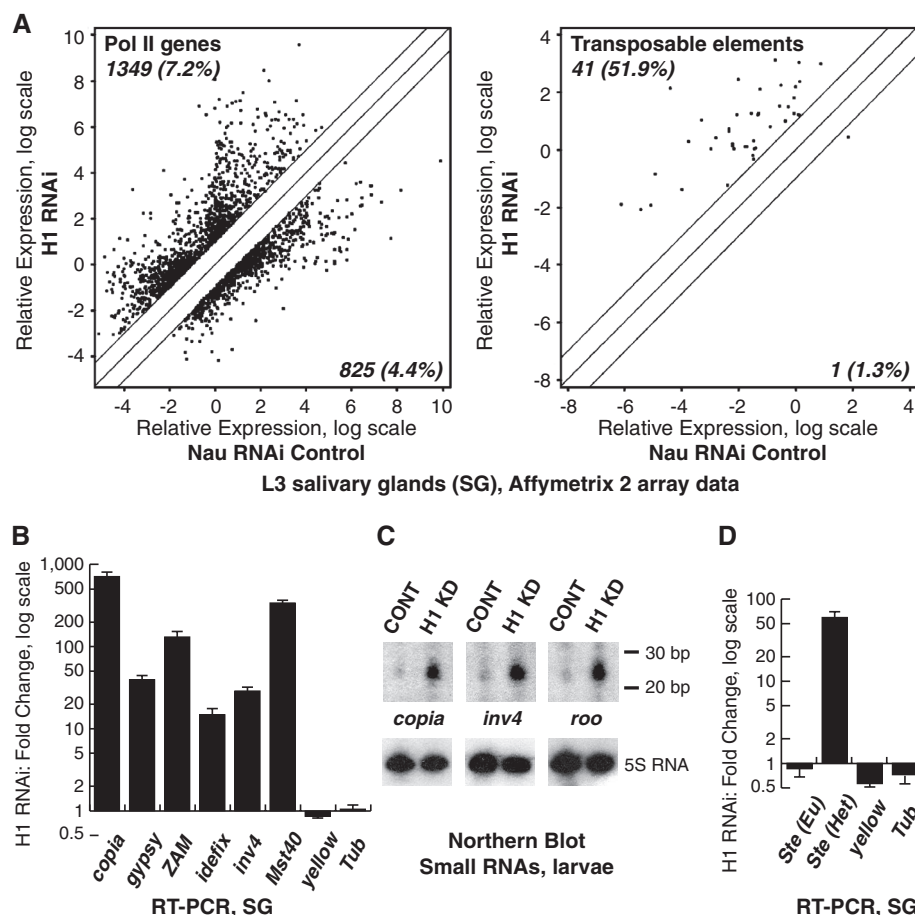
Repeat-associated small interfering RNA (rasiRNA) pathways are involved in the negative regulation of TEs (6, 7). In *Drosophila* germ cells, 24- to 28-nucleotide (nt) PIWI-interacting RNAs (piRNAs) are generated by the dicer activity of AGO3, whereas in ovarian soma piRNA-dependent silencing relies on the activity of PIWI, and in other types of somatic cells, TEs are repressed by 21-nt endo-siRNAs and AGO2 [reviewed in (8, 9)]. To test whether TE up-regulation in H1-depleted cells is due to decreased small RNA expression, we extracted total RNA from H1-depleted and control larvae and analyzed small RNAs homologous to *cop*, *inv*, *roo*, and *idex* transposable elements by Northern blotting. In each case, we observed a marked increase in the abundance of the corresponding small RNAs (Fig. 1C). We also used massive parallel sequencing to quantify small RNAs in SGs and ovaries from H1-depleted and control larvae. In each tissue we found that the majority of TE-specific small RNAs (both endo-siRNAs and piRNAs) are strongly up-regulated upon H1 depletion (table S2). Thus, activation of TE expression is not due to a decrease in the concentration of the repressive small RNAs.

TE insertions in *Drosophila* are thought to be mostly located in heterochromatin (10, 11) and

proximal heterochromatin-euchromatin transition zones (12). We hypothesized that H1 may silence TEs through its ability to regulate the activity of heterochromatin, with TEs responding differently to H1 depletion depending upon their insertion site. *Stellate* (*Ste*) (13) exhibits several features similar to TEs, with multiple tandem copies at two distinct loci, one euchromatic (*Eu Ste*) and the other in pericentric heterochromatin (*Het Ste*) (14). *Ste* expression is regulated by *Su(Ste)* encoding piRNAs that silence *Ste* (14, 15). Heterochromatic and euchromatic copies of *Ste* exhibit single-nucleotide polymorphisms that allow discrimination between transcripts originating from either locus. Depletion of H1 strongly up-regulates only *Het Ste* transcripts, whereas *Eu Ste* transcripts are not substantially affected (Fig. 1D). Although transcripts from both loci are negatively regulated by *Su(Ste)*-derived small RNAs, H1

specifically silences *Het Ste*, presumably through its role in regulation of heterochromatin function.

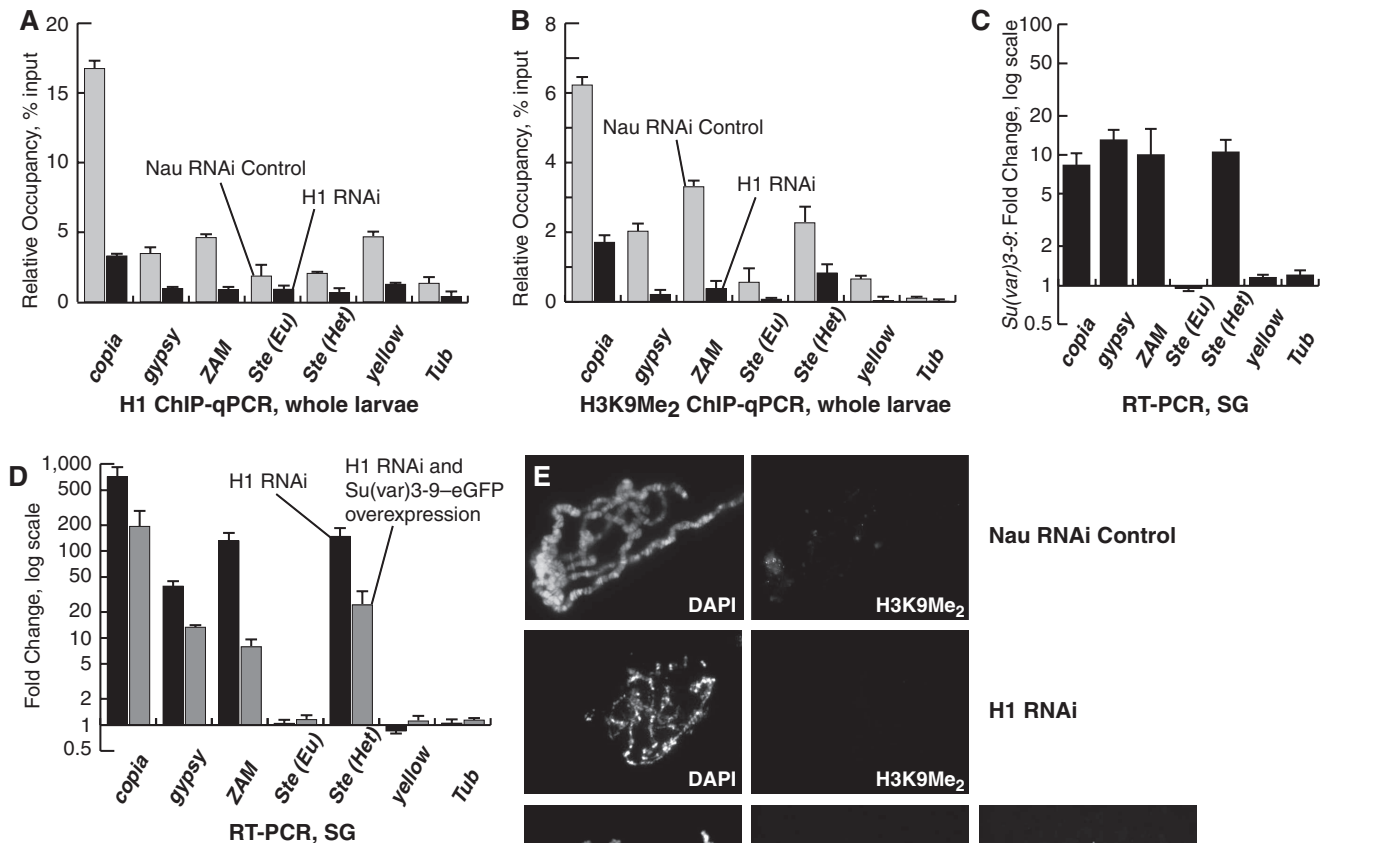
H1 depletion causes a reduction in dimethylation of histone H3 lysine 9 (H3K9Me<sub>2</sub>) (4). Quantitative chromatin immunoprecipitation (QChIP) in H1-depleted and control larvae at the regulatory regions of several TEs—including *cop*, *gypsy*, and *ZAM*—and at *Het Ste*, revealed a marked decline in the presence of H3K9Me<sub>2</sub> accompanying the loss of H1 (Fig. 2, A and B). Although H1 depletion also leads to reduced H3K9Me<sub>2</sub> at *Eu Ste* and other euchromatic loci like *yellow* (Fig. 2B), only heterochromatic loci are derepressed (Fig. 1, B and D), consistent with the existence of additional, H3 methylation-independent silencing mechanisms outside of heterochromatin. H3K9Me<sub>2</sub> modification is catalyzed primarily by the histone methyltransferase (HMT) *Su(var)3-9* (16). *Su(var)3-9* null mutation also leads to strong



**Fig. 1. *Drosophila* H1 represses repetitive elements.** (A) Transcript expression was examined by microarray analyses in H1-depleted and control (*Nau RNAi*) SGs (4). Signal intensities are shown for transcripts in control (x axis) versus H1-depleted (y axis) samples. The diagonal lines indicate equal expression level or a twofold change. Significantly affected transcripts above or below twofold threshold are indicated by dots. (Left) Signals for protein-coding gene probes; (right) signals for probes annotated as TEs. Numbers in the top left and bottom right corners represent percentages of transcripts that are up- or down-regulated above threshold, relative to the total number of probes (18,833 protein coding genes, 79 TEs). (B) TE transcripts in SGs were analyzed by QRT-PCR. Fold changes were calculated as a ratio of signals for H1-depleted samples to those for control samples and normalized to RP49. Standard deviations are from triplicate PCR reactions for three independent experiments. (C) RNA was extracted from H1-depleted (H1 KD) and control (CONT) larvae and (top) analyzed by Northern blot with TE-specific probes. (Bottom) Hybridization with the 5S RNA probe (loading control). (D) QRT-PCR of transcripts for euchromatic (*Eu*) and heterochromatic (*Het*) copies of *Ste* was analyzed as in (B).

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**Fig. 2. H1 represses repetitive elements in conjunction with Su(var)3-9.** (A) The occupancy of H1 in larval chromatin was measured by qChIP. The ordinate indicates the amounts of qChIP DNA samples relative to input DNA. All experiments were performed in triplicate. Error bars, standard deviation. (B) The occupancy of the H3K9Me<sub>2</sub> was measured by qChIP and presented as in (A). (C) QRT-PCR assays were performed in homozygous *Su(var)3-9*[6] and wild-type SGs. The data were analyzed as in Fig. 1B. (D) RNA was prepared from SGs from control, H1-depleted, and H1-depleted UAS:Su(var)3-9-eGFP larvae. QRT-PCR assays were performed as in (C).

Black bars, H1-depleted SGs; gray bars, H1-depleted UAS:Su(var)3-9-eGFP SGs. (E) SGs from control (top), H1-depleted (middle), and H1-depleted UAS:Su(var)3-9-eGFP (bottom) larvae were dissected, and polytene spreads were stained with 4',6'-diamidino-2-phenylindole (DAPI) and the indicated antibodies.

up-regulation of TEs and Het *Ste* but not Eu *Ste* (Fig. 2C), which suggests that H1 and Su(var)3-9 may function in concert. Although H3K9Me<sub>2</sub> is severely reduced in *Su(var)3-9* mutant larvae, H1 occupancy at repetitive sequences and its distribution in polytene chromosomes is not substantially affected (fig. S2, C to E). These results suggest that H1 acts upstream of Su(var)3-9 to regulate heterochromatin identity. If H1 and Su(var)3-9 cooperate to silence TEs, then overexpression of Su(var)3-9 might ameliorate the effects of H1 depletion. Indeed, overexpression of Su(var)3-9 partially reverses the activation of TE and Het *Ste* expression accompanying H1 depletion in SGs (Fig. 2D) and larvae (fig. S2F). In addition, Su(var)3-9 overexpression partially restores the decreased viability of flies caused by H1 depletion (table S3). Conversely, *Su(var)3-9* mutation strongly enhances the lethality caused by even moderate H1 depletion (table S3). Furthermore, Su(var)3-9 overexpression in the H1-depleted SG reinstates the H3K9Me<sub>2</sub> mark in pericentric heterochromatin (Fig. 2E). However, it does not rescue the global defects in

the morphology of polytene chromosomes, which suggests that the combined regulation of chromatin structure by H1 and Su(var)3-9 is directed specifically toward heterochromatin.

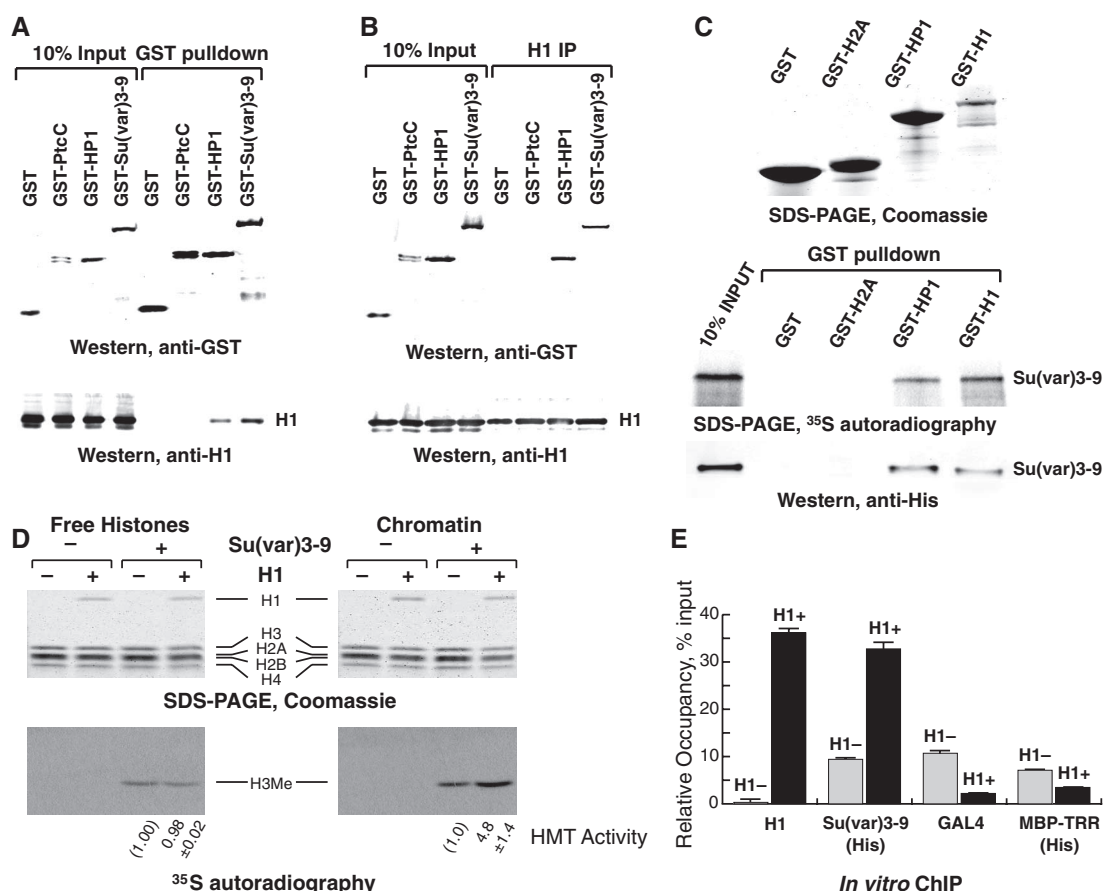
We next asked if H1 and Su(var)3-9 physically interact. A glutathione *S*-transferase (GST) fusion of Su(var)3-9 was expressed in *Drosophila* S2 cells, and immunoprecipitation (IP) of nuclear extracts with GST-specific antibody, followed by immunoblotting for H1, showed that endogenous H1 and GST-Su(var)3-9 associate (Fig. 3A). *Drosophila* H1 also interacts with heterochromatin protein 1 (HP1) (Fig. 3A), which parallels previous observations with their mammalian counterparts (17–19). Reciprocal IP with H1 antiserum confirmed the interaction of endogenous H1 with GST-Su(var)3-9 and GST-HP1 (Fig. 3B). Recombinant GST-H1 purified from bacteria also directly interacts with <sup>35</sup>S-labeled Su(var)3-9 translated in vitro and with purified recombinant Su(var)3-9-His<sub>6</sub> (Fig. 3C). The absence of binding between Su(var)3-9 and another histone protein (GST-H2A) shows that interaction of Su(var)3-9 with H1 is not due to the

high net positive charge of H1 or to a bridging artifact owing to contaminating nucleic acids.

To better understand the functional significance of this physical interaction, we studied Su(var)3-9 binding and its HMT activity toward chromatin reconstituted in vitro with and without H1. Recombinant Su(var)3-9-His<sub>6</sub> (fig. S3A) was assayed for HMT activity on free histones and oligonucleosomal templates (fig. S3, B and C). Su(var)3-9 can methylate histone H3 both when H3 is in the context of nucleosomes and in its dissociated native form (Fig. 3D). However, whereas the presence of H1 did not affect Su(var)3-9 activity toward H3 in solution, it strongly stimulated methylation of H3 assembled in chromatin (Fig. 3D). Thus, H1 does not stimulate the intrinsic enzymatic activity of Su(var)3-9, rather it promotes H3 methylation within the chromatin substrate. Furthermore, in vitro ChIP demonstrated a greater magnitude of Su(var)3-9 association with the H1-containing oligonucleosome substrate versus that with the H1-free substrate (Fig. 3E). In control ChIP experiments, the presence of H1 did not promote



**Fig. 3. H1 physically interacts with and recruits Su(var)3-9 to chromatin in vitro.** (A) GST fusion proteins were ectopically expressed in S2 cells and immunoprecipitated. The input and IP material was analyzed by immunoblotting with (top) GST- and (bottom) H1-specific antibodies. PTC, the C-terminal tail of Hedgehog receptor Patched (25) (negative control). (B) Reciprocal IP experiments with H1-specific antibody were performed as in (A). (C) Su(var)3-9 was expressed and <sup>35</sup>S-labeled by in vitro translation in reticulocyte lysates or purified as a 6His-tagged protein from bacteria. GST fusion proteins were expressed in *E. coli* and incubated with Su(var)3-9. The pulled-down material was examined by SDS-polyacrylamide electrophoresis (SDS-PAGE) and Coomassie staining (top), autoradiography (middle), or 6His-specific antibody immunoblotting (bottom). (D) Free histones (left) or reconstituted chromatin (right) with and without H1 were incubated with radioactive S-adenosylmethionine (SAM) in the presence or absence of recombinant Su(var)3-9-His<sub>6</sub>. H3 methylation was examined by autoradiography (bottom) and corrected for H3 loading (top, Coomassie). H3 methylation was quantified in two independent experiments; the average and standard deviation are shown at the bottom. (E) In vitro reconstituted chromatin with (H1+, black bars) or without H1 (H1-, gray



but, rather, strongly inhibited the occupancy of purified recombinant fusion of yeast GAL4 and herpes simplex virus VP16 proteins (GAL4-VP16), which can bind GAL4 sites present in the template, and MBP-TRR-His<sub>6</sub>, a fusion protein of Trithorax-related, H3K4-specific HMT (Fig. 3E, fig. S3A). These results indicate that H1 can specifically recruit Su(var)3-9 to chromatin where it methylates histone H3 in nucleosomes.

Our results indicate that *Drosophila* H1 and Su(var)3-9 work together in repressing the transcriptional activity of TEs and TE-like sequences in heterochromatin. Su(var)3-9 physically associates with H1 and is recruited to H1-containing chromatin, where it mediates H3K9 methylation. Considering the previously observed interactions between Su(var)3-9, HP1 and H3K9Me<sub>2/3</sub> [reviewed in (20)], H1 and HP1 (Fig. 3, A and B) (17–19, 21) and the physical interaction and joint activities of H1 and Su(var)3-9 reported here, we propose that these known heterochromatin effectors and components additionally require linker histone H1 for the establishment of heterochromatin identity and for repression of its genetic activity.

H1 is thought to be nearly ubiquitous in the genome, but several studies report its consistently higher abundance in heterochromatin (22–24). We

propose that higher concentrations of H1, equal in stoichiometry to nucleosomes, along extended chromatin domains may be essential to achieve its optimal function as a repressor, whereas substoichiometric or local deposition may only allow for a limited ability to repress genetic activity in euchromatin (Fig. 1A, left). In the future, it will be interesting to compare H1 abundance in various parts of the genome by physical fractionation of chromatin and to study the effects of H1 on activity of other chromatin-modifying enzymes.

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#### Supplementary Materials

www.sciencemag.org/cgi/content/full/340/6128/78/DC1  
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Tables S1 to S3  
References (26–35)

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# Translational Repression and eIF4A2 Activity Are Critical for MicroRNA-Mediated Gene Regulation

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MicroRNAs (miRNAs) control gene expression through both translational repression and degradation of target messenger RNAs (mRNAs). However, the interplay between these processes and the precise molecular mechanisms involved remain unclear. Here, we show that translational inhibition is the primary event required for mRNA degradation. Translational inhibition depends on miRNAs impairing the function of the eIF4F initiation complex. We define the RNA helicase eIF4A2 as the key factor of eIF4F through which miRNAs function. We uncover a correlation between the presence of miRNA target sites in the 3' untranslated region (3'UTR) of mRNAs and secondary structure in the 5'UTR and show that mRNAs with unstructured 5'UTRs are refractory to miRNA repression. These data support a linear model for miRNA-mediated gene regulation in which translational repression via eIF4A2 is required first, followed by mRNA destabilization.

MicroRNAs (miRNAs) are noncoding 21- to 25-nucleotide (nt) RNA molecules that in metazoans base-pair imperfectly with regions in target mRNAs [generally within the 3' untranslated region (3'UTR)] and repress the synthesis of the corresponding proteins (1). The mechanism for miRNA-mediated repression has remained elusive. Nevertheless, it is clear that miRNAs bind target mRNAs in complex with Argonaute proteins (Ago1 to Ago4 in humans). This complex then recruits one of the trinucleotide repeat-containing proteins (TNRC6A to TNRC6C), and this in turn leads to both translational repression and mRNA destabilization (2).

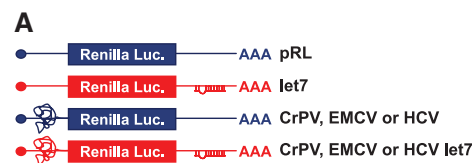
To determine whether mRNA degradation is a cause or consequence of translational inhibition, we used transfection of *in vitro* transcribed let-7 target reporter mRNAs. Translational repression of the target mRNA can clearly be observed as early as 30 to 60 min after transfection in the absence of miRNA-dependent mRNA destabilization (fig. S1), which is in agreement with (2–5). As deadenylation occurs on miRNA-repressed mRNAs, we considered the requirement for the polyadenylate [poly(A)] tail in miRNA-mediated repression. Transfection of mRNAs with and without a poly(A) tail showed that unadenylated mRNAs are efficiently repressed by miRNAs, although the addition of a poly(A) tail increased the magnitude of repression (fig. S2, A and B). Although increasing the length of the poly(A) tail results in more efficient translation rates (fig. S2, C and D), we observed maximal repression of the let-7-targeted mRNA with a relatively short poly(A) tail (fig. S2E). Furthermore, we observed equally efficient repression of an mRNA with a poly(A) tail of exactly (A)<sub>25</sub>(C)<sub>10</sub> as one with an (A)<sub>25</sub> tail, confirming that miRNA-mediated re-

pression is not dependent on deadenylation (fig. S2, F and G). Both of these mRNAs can bind one poly(A)-binding protein (PABP) molecule (6); however, the former cannot be deadenylated (fig. S2, H and I) (4, 7). Thus, the poly(A) tail, deadenylation, and mRNA degradation are not essential for miRNA-mediated translational repression, which is in agreement with other observations (2–6, 8).

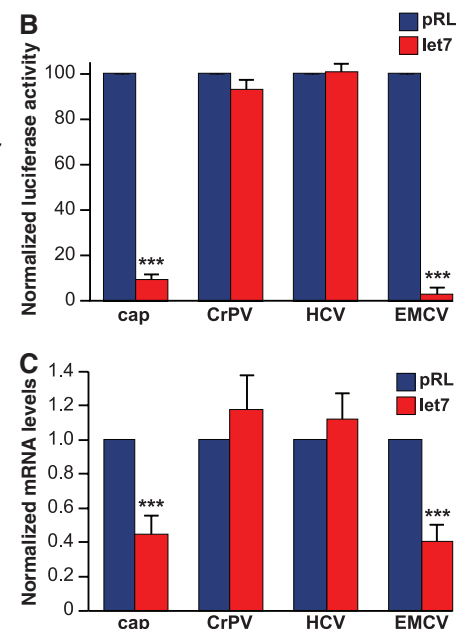
Evidence suggests that miRNAs exert their repressive activity at the translation initiation phase (9–12). To identify which translation initiation factors are required for repression, we appended the 5'UTRs of our reporter constructs with different internal ribosome entry site (IRES) sequences (Fig. 1A). The cricket paralysis virus (CrPV) IRES requires no initiation factors, binds directly to the ribosomal subunits, and promotes elongation (1). The hepatitis C virus (HCV) IRES

binds directly to the 40S ribosomal subunit and only requires eIF3, eIF2, eIF5, eIF5B, and Met-tRNA<sub>i</sub>, whereas the encephalomyocarditis virus (EMCV) IRES requires all initiation factors apart from eIF4E (1). A CrPV IRES mRNA containing let-7 target sites is not repressed, unlike the control mRNA (Fig. 1B), indicating that miRNA-mediated repression does not involve an elongation block, ribosome drop-off, or polypeptide degradation. The HCV IRES reporter was also refractory to miRNA-mediated repression, indicating that miRNA function is exerted before recruitment of the 60S ribosomal subunit, the eIF2-tRNA<sub>i</sub>Met ternary complex, eIF3, eIF5, or eIF5B. In contrast, translation of a let-7 reporter driven by the EMCV IRES was repressed as efficiently as was cap-dependent translation, indicating that miRNA-mediated repression is exerted after eIF4E and eIF4G recruitment. The observed effects are not restricted to let-7 (fig. S3, A and B) and the IRESs are functional (fig. S3, C to F). Destabilization of the IRES-containing reporter mRNAs only occurred on translationally repressed messages (Fig. 1C), even though nonrepressed mRNAs remain associated with the targeting miRNAs (fig. S4, A to C) and the RNA-induced silencing complex (RISC) (fig. S4, D and E). Additionally, we confirmed that the CrPV IRES reporter mRNA can undergo destabilization (fig. S4F). Thus, translational repression is the primary and required event for miRNA-mediated control, followed by mRNA destabilization. A number of factors may explain previous contradictory results involving miRNA-targeted IRES reporters, including the presence of a poly(A) tail and method of mRNA delivery (1).

Our analysis of IRES-dependent miRNA-mediated repression excluded many of the translation initiation factors that might be implicated in the repression mechanism. The initiation factors that are required for EMCV IRES— but not



**Fig. 1. Cap-dependent and EMCV IRES-driven translation is inhibited by let-7, whereas CrPV and HCV IRES-driven translation is not. (A)** Schematic representation of constructs used. Transcripts were either cap-dependent or driven by CrPV, HCV, EMCV IRESes ± eight repeats of let-7 target sites in the 3'UTR. **(B)** HeLa cells were transfected with the plasmids depicted in (A) together with a control firefly luciferase plasmid. Luciferase assays were performed after 48 hours. Luciferase activity presented is RLuc/Fluc, with the value for the constructs without miRNA target sites set at 100%. **(C)** RNA was isolated and analyzed by using quantitative reverse transcription polymerase chain reaction (RT-PCR) in parallel to (B). RLuc mRNA levels were calculated relative to Fluc mRNA levels.



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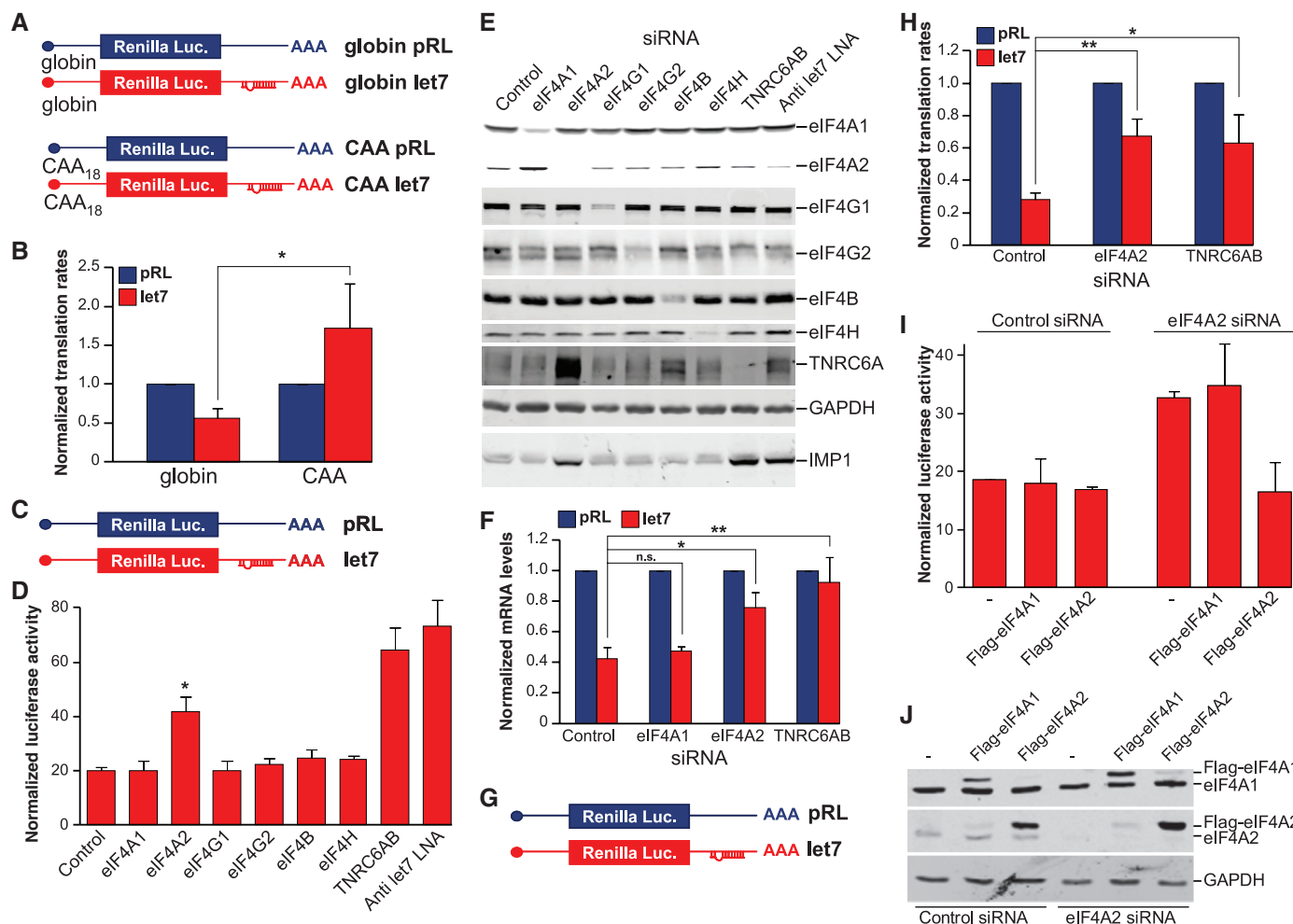
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HCV IRES-driven translation are all components of the eIF4F complex. To confirm that eIF4F function (which includes helicase activity and transcript scanning activity) is essential for repression, we transfected mRNAs carrying either the  $\beta$ -globin 5'UTR or an unstructured (CAA)<sub>18</sub> 5'UTR, which does not require unwinding (13),  $\pm$  let-7 target sites (Fig. 2A). The mRNA bearing the unstructured 5'UTR was refractory to miRNA-mediated repression and even showed a slight activation (Fig. 2B), confirming a requirement for eIF4F, which is in agreement with (11, 12).

To identify which of the implicated eIF4F components or associated factors are required for miRNA-mediated translational repression, small interfering RNAs (siRNAs) were used to knock down their expression, and DNA reporter con-

structs  $\pm$  let-7 target sites were used as readout. Only the knockdown of eIF4A2 affected miRNA-mediated repression (Fig. 2, C to E). This effect was not specific to the particular eIF4A2 siRNA used or restricted to one cell type (fig. S5, A to E). Depletion of both eIF4As did not change the extent of derepression (fig. S5, F and G). siRNA-depletion of eIF4A2 also increased the expression of a number of well-known endogenous miRNA targets (Fig. 2E and fig. S5H) (14). The knockdown of eIF4A2 or TNRC6A+B, but not eIF4A1, resulted in stabilization of reporter mRNA with let-7 target sites (Fig. 2F), again showing the requirement for translational repression for mRNA destabilization. Together, these results identify the eIF4F component eIF4A2 as the primary and key factor for miRNA-mediated gene silencing. To

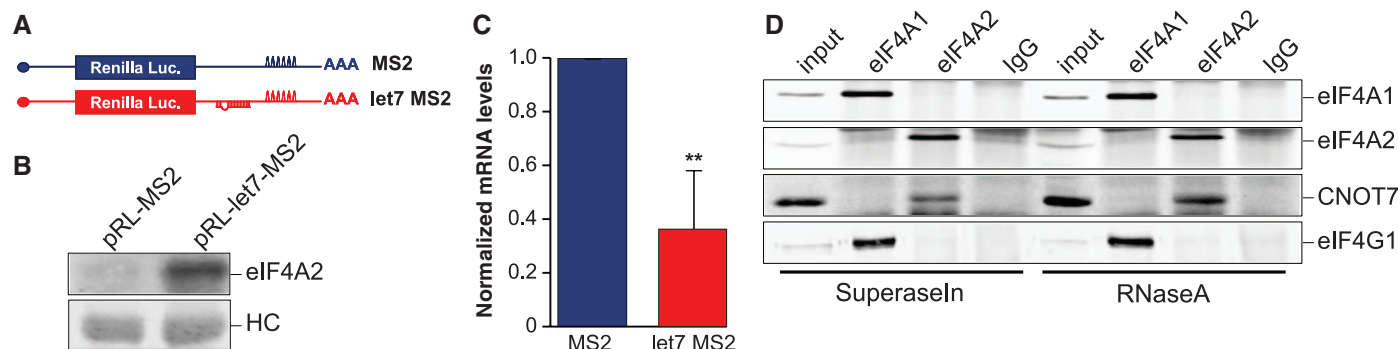
examine the early events in the miRNA repression pathway, before mRNA destabilization, the expression of either eIF4A2 or TNRC6A+B was knocked down, followed by transfection of in vitro transcribed mRNAs  $\pm$  let-7 target sites for 1 hour. At this time point, siRNA depletion of eIF4A2 or TNRC6A+B had similar effects on the miRNA target (Fig. 2, G and H, and fig. S5I). Transfection of a dominant negative form of eIF4A1 inhibited translation but did not affect the extent of miRNA-mediated regulation (fig. S6, A to C), whereas inhibition of both eIF4As with Pateamine A lead to a marked decrease in miRNA-mediated repression (fig. S6, D and E). Rescue experiments showed that after depletion of eIF4A2, only eIF4A2 and not eIF4A1 can restore repression (Fig. 2, I and J, and fig. S6, F and G),



**Fig. 2. eIF4A2 is the only component of eIF4F required for miRNA-mediated repression.** (A) Schematic representation of transcripts used for transfection with either the  $\beta$ -globin 5'UTR or an unstructured (CAA)<sub>18</sub> 5'UTR  $\pm$  two let-7 target sites. (B) HeLa cells were transfected with the in vitro transcribed mRNAs depicted in (A). Protein and RNA were harvested after 90 min. Translation rates were determined as a ratio of luciferase activity to mRNA levels (four biological repeats). (C) Constructs used for transfection in (D) to (F),  $\pm$  two let-7 target sites. (D) HeLa cells were transfected with the indicated siRNAs or an LNA against let-7 for 48 hours. Next, cells were transfected with the plasmid constructs depicted in (C) and the Firefly luciferase control. Luciferase activity was assayed after 24 hours. Values for control reporters were set at 100%. (E) HeLa cells were transfected in parallel with (D). Lysates were analyzed by means

of Western blot as indicated. IMP1 is a target of let-7. (F) HeLa cells were transfected as in (D), RNA isolated and analyzed by means of quantitative RT-PCR. RLuc mRNA levels were calculated relative to Fluc mRNA. (G) Transcripts used for transfection,  $\pm$  eight let-7 target sites. (H) HeLa cells were transfected with the indicated siRNAs. After 3 days, the cells were trypsinised, counted, and transfected with the in vitro transcribed mRNA depicted in (G). RNA and protein was collected after 60 min, and the translation rates were calculated (four biological repeats). (I) HeLa cells were transfected with the indicated siRNAs. Plasmids encoding the indicated Flag-tagged proteins were transfected after 24 hours, and reporters as depicted in (C) after another 24 hours. Luciferase assays were performed after a further 24 hours as in (D). (J) HeLa cells were transfected in parallel with (I). Lysates were analyzed by means of Western blot as indicated.





**Fig. 3. eIF4A2 is preferentially recruited to miRNA-targeted mRNAs and specifically associates with CNOT7, a component of the miRNA-repression machinery.** (A) Schematic representation of tethering constructs with 12 repeats of the MS2 hairpin within the 3'UTR  $\pm$  eight let-7 target sites. (B) MS2 immunoprecipitation of let-7-repressed mRNAs shows greater association with eIF4A2. HeLa cells were transfected with the plasmid constructs depicted in (A) together with a plasmid-expressing Flag-tagged MS2 coat

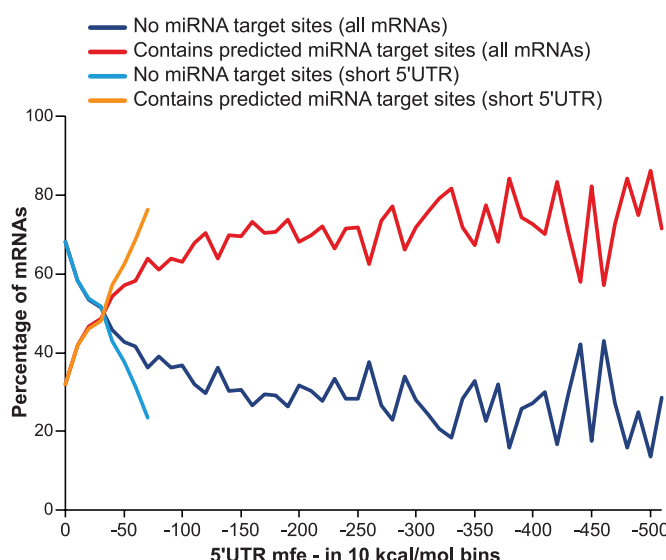
protein. After 48 hours, cells were lysed and subjected to Flag immunoprecipitation followed by Western blot analysis. HC, heavy chain. (C) Experiment as in Fig. 3B, analyzed by means of quantitative RT-PCR. (D) Immunoprecipitation from untransfected HeLa cell lysates using specific antibodies to eIF4A1 and eIF4A2 with ribonuclease (RNase) A or with RNase inhibitor (Supersasin). Recovered proteins were analyzed by using Western blot. Input, 10%.

confirming that eIF4A2 plays a critical role early in the repression pathway independently of eIF4A1.

eIF4A1 and eIF4A2 are both adenosine 5'-triphosphate (ATP)-dependent RNA helicases, believed to be involved in unwinding secondary structure within the 5'UTR of mRNAs, thus allowing the 40S ribosomal subunit to dock and scan toward the start codon (1). These highly related factors share 90% similarity at the protein level, with the major differences residing within the N-terminal domain. Although eIF4A1 is generally in excess of eIF4A2, they do not have identical functions (15). This prompted us to explore the possibility that eIF4A2, but not eIF4A1, was specifically interacting with micro-ribonucleoprotein complexes. To determine whether endogenous eIF4A2 associated with miRNA-repressed mRNAs, MS2 binding protein was used to immunopurify mRNAs containing MS2 hairpins  $\pm$  let-7 target sites within their 3'UTR. Western blotting was used to identify associated proteins. This experiment clearly shows that eIF4A2 specifically associates with let-7-repressed mRNAs (Fig. 3, A to C). Members of the TNRC6 protein family have been shown to interact with the Ccr4-NOT complex to induce both translational repression and mRNA deadenylation (16–19). Because a recent structural study has shown that cNOT1, a central component of this complex, contains a MIF4G fold similar to the eIF4A binding site of eIF4G (20), we investigated whether eIF4A2 interacts with the Ccr4-NOT complex. Indeed, immunoprecipitation of endogenous eIF4A2, but not of eIF4A1, reveals a specific RNA-independent association of eIF4A2 with CNOT7 (Fig. 3D). We also observed that eIF4G is preferentially associated with eIF4A1 (Fig. 3D), which was independently confirmed (fig. S6H).

Our results imply that mRNAs with functional miRNA-target sites in their 3'UTR would require structured 5'UTRs to enable repression. To examine this globally, 5'UTR structure prediction was performed by using RNAfold on the human RefSeq database. The mRNAs were grouped de-

**Fig. 4. mRNAs with miRNA target sites in their 3'UTR have a greater degree of secondary structure in the 5'UTR than do mRNAs without miRNA target sites.** 5'UTR sequences were analyzed by using RNAfold for potential secondary structure and grouped into 10 kcal/mol-wide minimum free-energy (mfe) bins. mRNAs within each bin were interrogated for conserved miRNA target sites. mRNAs with relatively short 5'UTRs (between 18 and 100 nt) were analyzed separately. Statistical significance for all mRNAs,  $P = 1.84 \times 10^{-197}$ ; for short 5'UTR mRNAs,  $P = 1.06 \times 10^{-22}$ .



pending on the presence or absence of conserved miRNA target sites in their 3'UTR as predicted with TargetScan (Fig. 4). mRNAs with miRNA target sites are more likely to have a greater degree of secondary structure within their 5'UTR. We reasoned that mRNAs with short 5'UTRs (less than 100 nt) should be even more dependent on secondary structure for miRNA-mediated activity. Indeed, we observed a stronger relationship between structure and the presence of conserved miRNA target sites within short 5'UTRs (Fig. 4).

Our data show that miRNAs induce gene silencing with translational repression occurring first and being required for subsequent mRNA destabilization. Moreover, we demonstrate that eIF4A2 and structure within the 5'UTR of target mRNAs are critical for miRNA-mediated repression. We show that eIF4A2 interacts with components of the repression machinery, namely the Ccr4-NOT complex. Interaction may be occurring via the MIF4G domain within cNOT1 (20) recruiting eIF4A2 and imposing translational re-

pression. The presence of eIF4A2 on a repressed mRNA via its interaction with the Ccr4-NOT complex would preclude the progression of initiation directed by eIF4G and eIF4A1, both for EMCV IRES- and, more importantly, cap-dependent translation.

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## Supplementary Materials

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References (21–38)

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# Archaeal (Per)Chlorate Reduction at High Temperature: An Interplay of Biotic and Abiotic Reactions

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Alfons J. M. Stams,<sup>1,5\*</sup> Bart P. Lomans<sup>6</sup>

Perchlorate and chlorate anions [(per)chlorate] exist in the environment from natural and anthropogenic sources, where they can serve as electron acceptors for bacteria. We performed growth experiments combined with genomic and proteomic analyses of the hyperthermophile *Archaeoglobus fulgidus* that show (per)chlorate reduction also extends into the archaeal domain of life. The (per)chlorate reduction pathway in *A. fulgidus* relies on molybdo-enzymes that have similarity with bacterial enzymes; however, chlorite is not enzymatically split into chloride and oxygen. Evidence suggests that it is eliminated by an interplay of abiotic and biotic redox reactions involving sulfur compounds. Biological (per)chlorate reduction by ancient archaea at high temperature may have prevented accumulation of perchlorate in early terrestrial environments and consequently given rise to oxidizing conditions on Earth before the rise of oxygenic photosynthesis.

Perchlorate and chlorate anions [together referred to as (per)chlorate] in the environment have long been considered as arising mainly from anthropogenic activities, namely the production of perchlorate-containing rocket propellants and ammunitions followed by environmental pollution (1). However, recent findings indicate that perchlorate is continuously formed naturally in the atmosphere, with proposed mechanisms ranging from photochemically triggered processes (2) to electrical discharge-based reactions and ozone oxidation of chlorides (3). Such natural sources make perchlorate an ubiquitous compound on Earth, although sizeable accumulations tend to be limited to certain arid environments, like the Atacama desert in Chile (4). Perchlorate deposits also exist on Mars (5). It has been proposed that the lack of perchlorate accumulation elsewhere on Earth might be due to microbial activity (4), which is supported by the widespread occurrence of bacteria that can use perchlorate and chlorate as terminal electron acceptor for growth (6).

(Per)chlorate-reducing bacteria described so far belong mainly to the bacterial phylum of

Proteobacteria (7). The widely accepted pathway of biological (per)chlorate reduction consists of a two-step reduction from perchlorate, via chlorate to chlorite, followed by a dismutation to molecular oxygen and chloride (8). This metabolism relies on the action of a perchlorate reductase (Pcr) and a chlorate reductase (Clr), two functions that in perchlorate reducers are often performed by a single enzyme (9); it further requires a chlorite dismutase (Cld) to form molecular oxygen and chloride. Microbial formation of molecular oxygen under anaerobicity is a biochemical rarity.

Because the reported mechanisms for natural perchlorate generation on Earth seem to have existed already during preanthropogenic times (1, 2), the appearance of biological reduction of (per)chlorate may have been an important event in Earth's history. One indication of an ancient origin for reduction of (per)chlorate would be its occurrence in microorganisms that thrive in environments resembling those of early Earth. *Archaeoglobus fulgidus*, a hyperthermophilic archaeon, fulfills this criterion. *A. fulgidus* was isolated from marine hot vents close to Vulcano island in Italy (10) but has since then been found in many extreme subsurface environments, such as hot oil reservoirs or geothermal formations (11, 12). It is considered to be a major contributor to sulfate reduction and sulfide formation at high temperature. *A. fulgidus* strain VC-16 is the best-studied sulfate-reducing archaeon. Its genome contains many oxidoreductases genes with unknown function (13).

We demonstrate that *A. fulgidus* strain VC-16 as well as *A. fulgidus* strain Z (14) (fig. S1) can grow with perchlorate or chlorate as electron ac-

ceptors (Fig. 1 and table S1). Our finding extends (per)chlorate reduction into the archaeal domain of life and high temperature environments.

Enzyme assays showed chlorate-reducing activity in cell-free extracts and suspensions of *A. fulgidus* VC-16 that were comparable with activities found in some mesophilic (per)chlorate-reducing bacteria (table S2). An activity toward perchlorate could not clearly be identified, which is similar to an earlier study on the (per)chlorate reductase of *Azospira oryzae* strain GR-1 (9). No chlorite dismutase activity could be detected, either. Consistent with this, no genes similar to known chlorite dismutases were identified in the genome of *A. fulgidus*. On the other hand, no accumulation of chlorite was observed, suggesting an alternative mechanism of chlorite conversion.

Perchlorate- and chlorate-grown cells of *A. fulgidus* are still able to use sulfate as electron acceptor. When sulfate and perchlorate are present together in the cultures, both are used simultaneously, whereas sulfate reduction is delayed if chlorate is present (Fig. 1, C and D). Simultaneous perchlorate and sulfate reduction in a single culture is intriguing from an energetic viewpoint. The midpoint potentials of redox couples involved in (per)chlorate reduction are high, whereas those involved in sulfate reduction are low [for  $\text{ClO}_4^-/\text{ClO}_3^-$ , redox potential ( $E^0$ ) = +0.788 V;  $\text{ClO}_3^-/\text{ClO}_2^-$   $E^0$  = +0.709 V;  $\text{ClO}_2^-/\text{Cl}^-$   $E^0$  = +1.199 V versus  $\text{SO}_4^{2-}/\text{HSO}_3^-$   $E^0$  = −0.516 V;  $\text{HSO}_3^-/\text{HS}^-$   $E^0$  = −0.110 V]. During the reduction of (per)chlorate, the redox potential is locally increasing, but the overall redox state remains low in all the cultures throughout the entire experiment ( $\leq -200$  mV), indicated by resazurin in the medium and a redox electrode.

Sulfide is normally omitted from media when growing (per)chlorate reducers but is used to establish the low redox potential required for growth of strict anaerobes like *A. fulgidus*. Similar to sulfate reduction, (per)chlorate reduction in *A. fulgidus* requires reduced conditions that are established by the addition of sulfide to the medium and even pronounced by sulfide formation if sulfate is present as well (Fig. 1, C and D).

Proteome analysis of cells grown with either perchlorate or chlorate shows that, in comparison with sulfate-grown cells, there is an increased abundance of a large number of proteins that are associated with redox and oxygen stress (table S3). We hypothesize that in the presence of (per)chlorate these proteins play a crucial role in creating and maintaining a low intracellular redox potential, which is a basic requirement for dissimilatory sulfate reduction. The differential expression of

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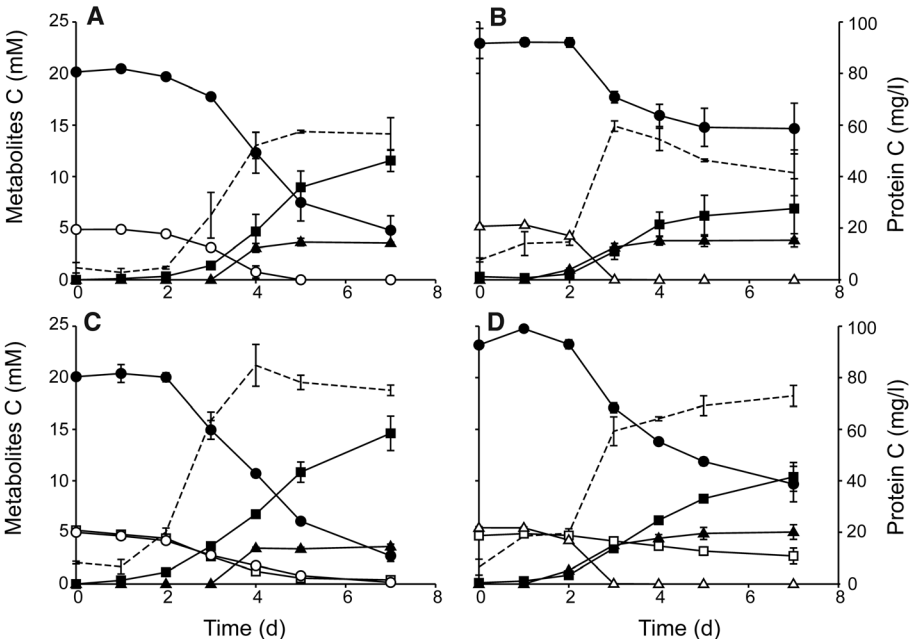
stress proteins reflects differences in redox-stress conditions. Potentially destructive redox stress is also indicated by the increased abundance of enzymes involved in cell repair (table S3). The higher Gibbs free energy change when coupling the oxidation of a substrate to (per)chlorate instead of sulfate (table S4) was not visible in terms of enhanced growth rate and/or higher growth yield of *A. fulgidus* strain VC-16. The mass balances at the three growth conditions resulted in similar protein concentrations as a measure of biomass (table S1). These growth yields reflect high maintenance costs when growing with (per)chlorate.

The proteome of VC-16 cells grown with (per)chlorate also contains highly abundant proteins encoded by three gene clusters that are annotated as molybdopterin oxidoreductases (locus tags of cluster I are AF0157 to AF0160; cluster II, AF0173 to AF0176; and cluster III, AF2384 to AF2386) (Table 1).

Cluster II is the best candidate for the reduction of (per)chlorate. Its catalytic subunit encoded by AF0176 has 31% identity (on protein level) and a query coverage of 98% with the alpha subunit of the characterized (per)chlorate reductase of *Dechloromonas agitata* (15). Cluster I and cluster III are most likely involved in the metabolism of sulfur-based substrates: Cluster I carries a conserved domain in its  $\alpha$  subunit for a tetrathionate reductase (cd02758), whereas that of cluster III carries a conserved domain for thiosulfate, sulfur, and polysulfide reductases (cd02755) (16, 17).

Structure-based modelling suggested that AF0176 possibly is the catalytic subunit of a periplasmic NarG-type nitrate reductase (18). However, NarG key residues found in AF0176 can often be identified in the  $\alpha$  subunit of characterized perchlorate reductases (PcrA) as well (fig. S2). Thus far, no reports mention the ability of *Archaeoglobus* species to respire with nitrate. Nitrate reductase and Pcr both belong to the type II dimethyl sulfide family of enzymes and share the conserved domain cd02750, indicating the high level of similarity between these enzymes. In addition, Pcrs are known to reduce nitrate as well (9).

The apparent involvement of the three molybdopterin oxidoreductases in (per)chlorate reduction suggests the following model. (Per)chlorate reduction performed by AF0174-0176 results in the formation of chlorite, which reacts abiotically with sulfide. The reactivity of sulfide with chlorite triggers a cascade of subsequent reactions forming several higher-oxidized sulfur compounds (19, 20). Depending on the conditions in the medium, all sulfur might eventually be converted to sulfate (fig. S3). The accumulation of oxidized sulfur fractions requires the action of enzymes regenerating reduced sulfur compounds to continuously detoxify chlorite. These conversions can be catalyzed by AF0157-0159, a tetrathionate reductase-like enzyme, and AF2384-2386, an enzyme similar to thiosulfate, sulfur, and polysulfide reductases. Our findings give rise to a hypothesis in which (per)chlorate



**Fig. 1. (Per)chlorate reduction by *A. fulgidus* strain VC-16.** Experiments were performed with [(C) and (D)] and without [(A) and (B)] addition of sulfate as an alternative electron acceptor. The reduction of perchlorate (open circle), chlorate (open triangle), and sulfate (open square) is coupled to the oxidation of lactate (solid circle), producing acetate (solid square) and formate (solid triangle). Growth is represented by the protein concentration (dashed line) plotted against the secondary y axis (right). Inocula used were exponentially growing perchlorate- [(A) and (C)] and chlorate-reducing [(B) and (D)] cultures, respectively. In the absence of either electron acceptor or electron donor (lactate), no growth and substrate conversion were observed (table S1 and fig. S4); means  $\pm$  ranges (error bars),  $n = 2$ .

**Table 1. Expression of key proteins involved in (per)chlorate reduction by *A. fulgidus* strain VC-16.** Growth was performed with perchlorate, chlorate, and sulfate as electron acceptors. Columns indicate biological duplicates. Protein accession numbers are in parentheses. The full set can be found in the supplementary materials (table S3).

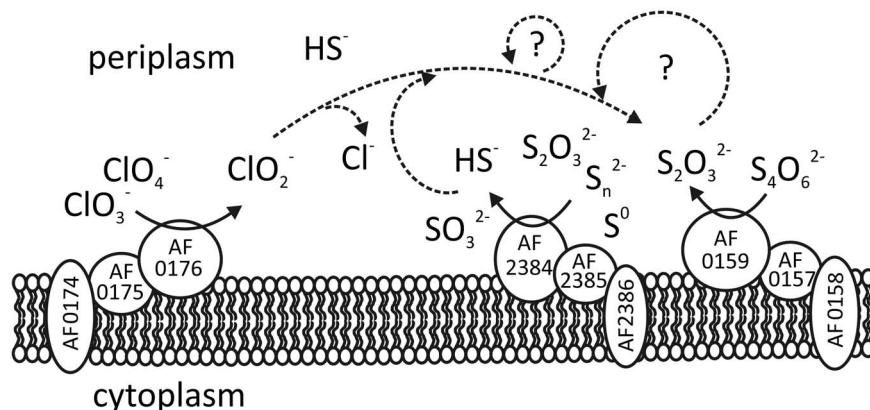
| Function                                                   | Spectral counts |      |          |      |         |      |
|------------------------------------------------------------|-----------------|------|----------|------|---------|------|
|                                                            | Perchlorate     |      | Chlorate |      | Sulfate |      |
| Molybdo-oxidoreductase I<br>( $\alpha$ subunit: AF0159)    | 568             | 712  | 261      | 300  | 20      | 16   |
| Molybdo-oxidoreductase II<br>( $\alpha$ subunit: AF0176)   | 1039            | 1190 | 640      | 766  | 112     | 69   |
| Molybdo-oxidoreductase III<br>( $\alpha$ subunit: AF02384) | 1305            | 1392 | 1703     | 2154 | 349     | 190  |
| Adenosine triphosphate sulfurylase (AF1667)                | 952             | 1033 | 741      | 834  | 1769    | 1515 |
| Adenylylsulfate reductase<br>( $\alpha$ subunit: AF1670)   | 2399            | 1906 | 1856     | 1375 | 3121    | 2871 |
| Sulfite reductase<br>( $\alpha$ subunit: AF0423)           | 255             | 328  | 265      | 450  | 317     | 283  |

reduction by *A. fulgidus* relies strictly on a sulfur-based cycle that is driven by both biotic and abiotic processes (Fig. 2). In nature these reduced conditions might also be facilitated by coexisting sulfate-reducing organisms or alternative reductants.

In our model, complete conversion of sulfide to sulfate is not essential, but the enzymes needed to activate and reduce sulfate are constitutively present (21) (Table 1). The chemical destruction of chlorite formed during biological (per)chlorate reduction by *A. fulgidus* is thus an alternative to biological dismutation.

Because the (per)chlorate reduction mechanism in *A. fulgidus* does not involve chlorite dismutase, a key enzyme in known (per)chlorate-reducing bacteria, the coupling of biotic processes to the abiotic removal of the produced chlorite is a necessity. The fact that a strict anaerobic archaeon is able to reduce and grow with (per)chlorate as electron acceptor suggests that this metabolism may be more widespread in the prokaryotic world. Furthermore, if perchlorate was produced in Earth's early atmosphere, biological mechanisms using this compound may have





**Fig. 2. Proposed pathway and enzymatic machinery for the reduction of (per)chlorate coupled to sulfur compounds in *A. fulgidus*.** Reductases involved in the reduction of (per)chlorate (AF0174 to AF0176) and tetrathionate (AF0157 to AF0159) and an enzyme similar to thiosulfate-, sulfur-, and polysulfide reductases (AF2384 to AF2386) are shown. Interactions between biological and chemical processes are illustrated by solid and dashed lines, respectively.

evolved at a very early time, potentially even before oxygen-generating photosynthesis. The use of (per)chlorate together with other compounds such as nitrogen-oxo compounds may depict the first entry of highly oxidative compounds into microbial metabolism and thus could have contributed to the rise of life adapted to more oxidizing conditions on Earth (22).

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#### Supplementary Materials

www.sciencemag.org/cgi/content/full/340/6128/85/DC1  
Materials and Methods  
Figs. S1 to S5  
Tables S1 to S4  
References (23–34)

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## Influence of HLA-C Expression Level on HIV Control

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A variant upstream of human leukocyte antigen C (HLA-C) shows the most significant genome-wide effect on HIV control in European Americans and is also associated with the level of HLA-C expression. We characterized the differential cell surface expression levels of all common HLA-C allotypes and tested directly for effects of HLA-C expression on outcomes of HIV infection in 5243 individuals. Increasing HLA-C expression was associated with protection against multiple outcomes independently of individual HLA allelic effects in both African and European Americans, regardless of their distinct HLA-C frequencies and linkage relationships with HLA-B and HLA-A. Higher HLA-C expression was correlated with increased likelihood of cytotoxic T lymphocyte responses and frequency of viral escape mutation. In contrast, high HLA-C expression had a deleterious effect in Crohn's disease, suggesting a broader influence of HLA expression levels in human disease.

Variation within the human leukocyte antigen (HLA) class I genes of the major histocompatibility complex (MHC) has

the greatest impact on outcome after HIV infection relative to the rest of the genome (1–3). A single-nucleotide polymorphism 35 kb up-

stream of the HLA-C locus (rs9264942) showed the most significant association with viral load (VL) control in a recent genome-wide association study (GWAS) (2) and the second most significant association in an earlier HIV GWAS where VL at set point was considered (3). We previously reported that rs9264942 genotype correlated with the level of HLA-C cell surface protein expression on primary T cells from European Americans (4). Whether rs9264942 is associated with the outcome of HIV infection because it marks HLA-C expression levels or individual HLA alleles that affect HIV remains unknown (4, 5).

If HLA-C expression levels have a direct influence on HIV control, then we expect to observe the effect across ethnic groups, despite their distinct HLA-C allele frequencies and linkage disequilibrium (LD) relationships with HLA-A and -B alleles (6). Unlike in European Americans, rs9264942 is in poor LD with HLA-C alleles in African Americans and does not mark expression levels of HLA-C or associate with HIV control in large studies (2, 7) (fig. S1). We determined the expression levels of individual HLA-C allotypes in African Americans, which showed a continuous distribution (Fig. 1), in order to test an effect of HLA-C expression level on HIV control.

HLA-C alleles that are present in both African and European Americans show the same

relative expression levels in the two populations (4) (Fig. 1). Indeed, a highly significant correlation was observed in both 50 European-American and 50 African-American donors when comparing the observed HLA-C expression levels with expected levels based on mean expression of each HLA-C allele observed in an independent group of 150 African Americans (fig. S2). After confirming that HIV infection does not alter HLA-C expression (fig. S2), we used the mean expression of individual HLA-C alleles measured in all 200 African-American donors to assign HLA-C expression levels to each HIV-infected subject based on their HLA-C genotypes (8). An effect of HLA-C expression level was tested, along with all individual HLA class I alleles, in chronically infected subjects using logistic regression with stepwise selection. HLA-C expression level showed a significant independent association with HIV control in an analysis of 2527 European-American patients [ $P = 1 \times 10^{-7}$ , odds ratio (OR) = 0.52] (Table 1), where OR for HLA-C expression represents the protection conferred by a difference of

100 higher median fluorescence intensity (MFI) expression units (Fig. 1). The average difference in expression of 224 units between Cw\*07 and Cw\*06 homozygotes, for example, would correspond to an OR of 0.24.

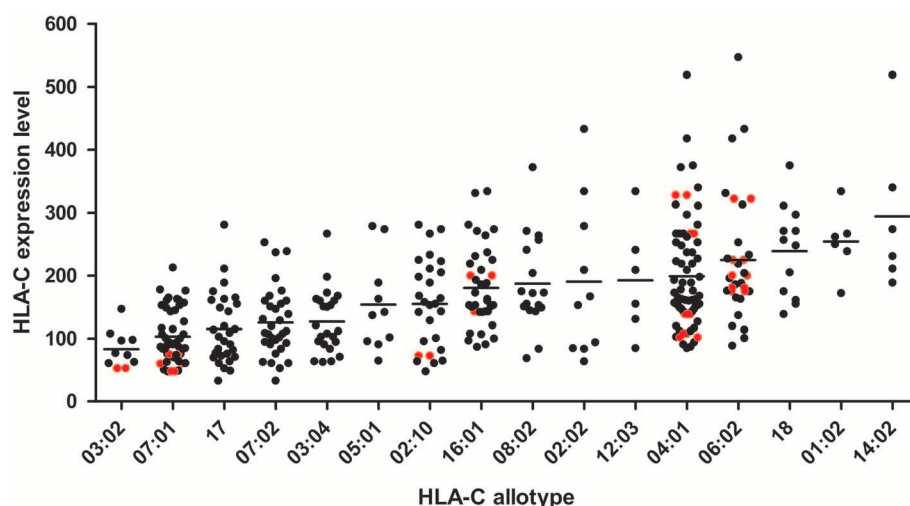
The protective effect of high HLA-C expression level in European Americans was mirrored in an analysis of 1209 African-American patients, where expression had the third most significant independent effect ( $P = 8 \times 10^{-6}$ , OR = 0.61) (Table 1) after HLA-B\*57:03 and B\*81, two well-documented protective alleles in cohorts of African descent (7, 9). Similar results were observed when mean VL was considered as a continuous variable (table S1). Although our African-American population is admixed to some extent with European genotypes, it is clearly distinct from our European-American cohorts (fig. S1). The effect of HLA-C expression level persisted when increasing the stringency at which covariates were considered to have independent effects, and permutation analyses indicated that the distribution of HLA-C allelic expression values observed are unlikely to correlate with HIV control by chance (8).

HLA-C expression level showed the most significant effect on progression from seroconversion to CD4 < 200 in a cohort of 1069 subjects [ $P = 1 \times 10^{-6}$ , hazard ratio (HR) = 0.67 for a difference of 100 MFI expression units] relative to all individual HLA alleles (Table 2). Thus, the continuous distribution of HLA-C allele expression levels correlates with multiple outcomes of HIV infection, tested in three distinct cohorts: longitudinal VL in European Americans, longitudinal VL in African Americans, and progression to CD4 < 200 in a separate cohort of European and African Americans combined (8). Use of regression models that include all HLA class I alleles as covariates, along with consistent effects across African and European Americans, strongly point

to HLA-C expression level as a major independent contributor to control of HIV.

A direct genetic effect of HLA-C expression level on HIV control predicts that more highly expressed HLA-C allotypes would confer greater immune pressure on the virus, which can be assessed by measuring the strength of association between a given HLA-C allele and corresponding mutations in the viral genome. HLA class I genotypes and nearly full-length HIV sequences were generated in a cohort of 1888 clade B chronically infected patients (the majority Caucasian) who were treatment naïve. A phylogenetically corrected logistic regression approach was used to estimate the probability of postinfection escape mutations and determine their independent associations with HLA alleles present (8, 10, 11). Viral sequence mutations showing significant independent associations with HLA-C were then filtered for predicted epitopes that fit the biochemical features of the HLA-C allele involved (12). Twenty-two mutations were identified, representing escape at 12 epitopes that associated with 7 HLA-C alleles (table S3). Median log odds ratios were calculated for mutations at all epitopes associated with the same HLA-C allele to determine the strength of selection conferred by each of these 7 HLA-C alleles (table S3). A strong positive correlation between HLA-C expression level and the strength of selection pressure conferred on the virus by the allele was observed ( $r = 0.87$ ,  $P = 0.005$ ) (Fig. 2A), indicating that higher HLA-C expression exerts greater selection on HIV. This finding is consistent with a study of Han Chinese that identified six viral mutations associated with HLA-C alleles that occurred more commonly in 99 patients with the rs9264942 genotype CC than in 63 patients with the TT genotype (13).

We analyzed cytotoxic T lymphocyte (CTL) responses to overlapping peptides spanning the



**Fig. 1. The distribution in expression levels of HLA-C allotypes present in African Americans.** Peripheral blood CD3<sup>+</sup> cells from 200 healthy donors were analyzed by flow cytometry for HLA-C expression level using the monoclonal antibody DT9. MFI of HLA-C staining is plotted twice for each donor (i.e., once for each HLA-C allele present), with HLA-C homozygous individuals marked in red. Expression level correlates significantly with HLA-C allotypes in analysis of variance ( $P = 5 \times 10^{-21}$ ).

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**Table 1. HLA-C expression level affects control of HIV viral load in European and African Americans.** Antiretroviral therapy (ART)–naïve HIV patients were categorized as controllers (<2000 viral copies/ml plasma) or noncontrollers (>10,000 viral copies/ml plasma) on the basis of longitudinal mean VL in chronic

infection. Effects of HLA-C expression level as a continuous variable and all individual HLA class I alleles with phenotypic frequency  $\geq 2\%$  were tested by logistic regression with stepwise selection. Significance (*P*), OR, and 95% confidence interval (CI) are reported for all covariates showing independently significant effects.

| European Americans ( <i>n</i> = 2527) |                                      |             |             |             | African Americans ( <i>n</i> = 1209) |                                      |             |             |             |
|---------------------------------------|--------------------------------------|-------------|-------------|-------------|--------------------------------------|--------------------------------------|-------------|-------------|-------------|
| Covariate                             | <i>P</i>                             | OR          | 95% CI      |             | Covariate                            | <i>P</i>                             | OR          | 95% CI      |             |
| B*57:01                               | $1 \times 10^{-19}$                  | 0.13        | 0.09        | 0.21        | B*57:03                              | $2 \times 10^{-24}$                  | 0.13        | 0.09        | 0.20        |
| C*04:01                               | $8 \times 10^{-14}$                  | 3.12        | 2.32        | 4.21        | B*81                                 | $2 \times 10^{-8}$                   | 0.21        | 0.12        | 0.37        |
| A*01:01                               | $2 \times 10^{-10}$                  | 2.21        | 1.73        | 2.82        | <b>HLA-C expression</b>              | <b><math>8 \times 10^{-6}</math></b> | <b>0.61</b> | <b>0.49</b> | <b>0.76</b> |
| B*07:02                               | $2 \times 10^{-9}$                   | 2.36        | 1.78        | 3.13        | B*35:01                              | $1 \times 10^{-5}$                   | 2.92        | 1.80        | 4.74        |
| <b>HLA-C expression</b>               | <b><math>1 \times 10^{-7}</math></b> | <b>0.52</b> | <b>0.41</b> | <b>0.67</b> | B*45:01                              | $3 \times 10^{-5}$                   | 4.28        | 2.16        | 8.50        |
| A*02:01                               | $4 \times 10^{-7}$                   | 1.68        | 1.38        | 2.06        | A*23:01                              | $1 \times 10^{-4}$                   | 2.05        | 1.43        | 2.96        |
| B*27:05                               | $2 \times 10^{-5}$                   | 0.48        | 0.34        | 0.67        | B*58:02                              | $3 \times 10^{-3}$                   | 2.54        | 1.37        | 4.73        |
| C*06:02                               | $2 \times 10^{-5}$                   | 2.65        | 1.69        | 4.15        | B*58:01                              | $4 \times 10^{-3}$                   | 0.49        | 0.30        | 0.79        |
| B*13:02                               | $4 \times 10^{-4}$                   | 0.39        | 0.23        | 0.65        | A*36:01                              | $6 \times 10^{-3}$                   | 3.22        | 1.40        | 7.38        |
| C*16:01                               | $6 \times 10^{-4}$                   | 2.12        | 1.38        | 3.26        | B*39:10                              | $1 \times 10^{-2}$                   | 0.34        | 0.14        | 0.78        |
| A*24:02                               | $9 \times 10^{-4}$                   | 1.58        | 1.20        | 2.07        | B*53:01                              | $3 \times 10^{-2}$                   | 1.48        | 1.04        | 2.11        |
| C*01:02                               | $3 \times 10^{-3}$                   | 1.90        | 1.24        | 2.91        |                                      |                                      |             |             |             |
| B*52:01                               | $4 \times 10^{-3}$                   | 0.49        | 0.30        | 0.79        |                                      |                                      |             |             |             |
| A*26:01                               | $4 \times 10^{-3}$                   | 1.80        | 1.20        | 2.70        |                                      |                                      |             |             |             |
| B*40:01                               | $6 \times 10^{-3}$                   | 1.69        | 1.16        | 2.45        |                                      |                                      |             |             |             |
| B*38:01                               | $7 \times 10^{-3}$                   | 2.02        | 1.22        | 3.34        |                                      |                                      |             |             |             |
| C*05:01                               | $1 \times 10^{-2}$                   | 1.43        | 1.09        | 1.89        |                                      |                                      |             |             |             |
| B*18:01                               | $1 \times 10^{-2}$                   | 1.56        | 1.10        | 2.23        |                                      |                                      |             |             |             |
| B*58:01                               | $2 \times 10^{-2}$                   | 0.49        | 0.27        | 0.90        |                                      |                                      |             |             |             |
| B*14:02                               | $3 \times 10^{-2}$                   | 0.67        | 0.46        | 0.96        |                                      |                                      |             |             |             |
| B*51:01                               | $4 \times 10^{-2}$                   | 1.47        | 1.01        | 2.13        |                                      |                                      |             |             |             |
| B*55:01                               | $5 \times 10^{-2}$                   | 2.07        | 1.02        | 4.21        |                                      |                                      |             |             |             |

entire HIV clade C proteome, that were measured by enzyme-linked immunosorbent spot assay in 1010 HIV subtype C–infected South African subjects (14, 15). HLA-associated CTL responses were determined by testing the log OR of observing a response to each peptide in the presence versus absence of every HLA class I allele, using a decision tree with Fisher’s exact test to discriminate associations due to linked HLA alleles (8). CTL responses restricted by all of the 14 two-digit HLA-C alleles present in our population were observed, comprising 71 distinct associations between a given peptide and a given HLA-C allele (table S4). Median log ORs were calculated for responses to all peptides associating with the same HLA-C allele. A strong correlation between the level of HLA-C allele expression and average strength of association with CTL response to HIV was observed ( $r = 0.73$ ,  $P = 0.002$ ) (Fig. 2B), such that HIV peptides presented by higher expressed HLA-C alleles were more likely to elicit CTL responses than peptides presented by low expression HLA-C allotypes.

Of the total 60 peptides associated with HLA-C–restricted CTL responses, 8 peptides associated with responses restricted by two different HLA-C allotypes and 4 peptides associated with responses restricted by three different HLA-C allotypes. This permits a total of 20 separate comparisons of the strength of response to a single peptide between two HLA-C allotypes that differ in their level of expression (table S5). A significant correlation was observed between the difference in

**Table 2. HLA-C expression level affects progression to early AIDS outcomes.** HLA-C expression level as a continuous variable and all HLA class I alleles with phenotypic frequency  $\geq 2\%$  were tested by stepwise selection in a Cox model, using 1069 ART-naïve patients with known times for progression from seroconversion to CD4 < 200. Subjects comprised 783 European and 286 African Americans, with ethnicity used as a covariate. This was justified because stratification by ethnicity indicated a uniform strength of effects on progression across European and African Americans (table S2), which was also apparent in the larger longitudinal VL cohorts (Table 1). Independently significant covariates are reported with significance (*P*), HR, and 95% CI.

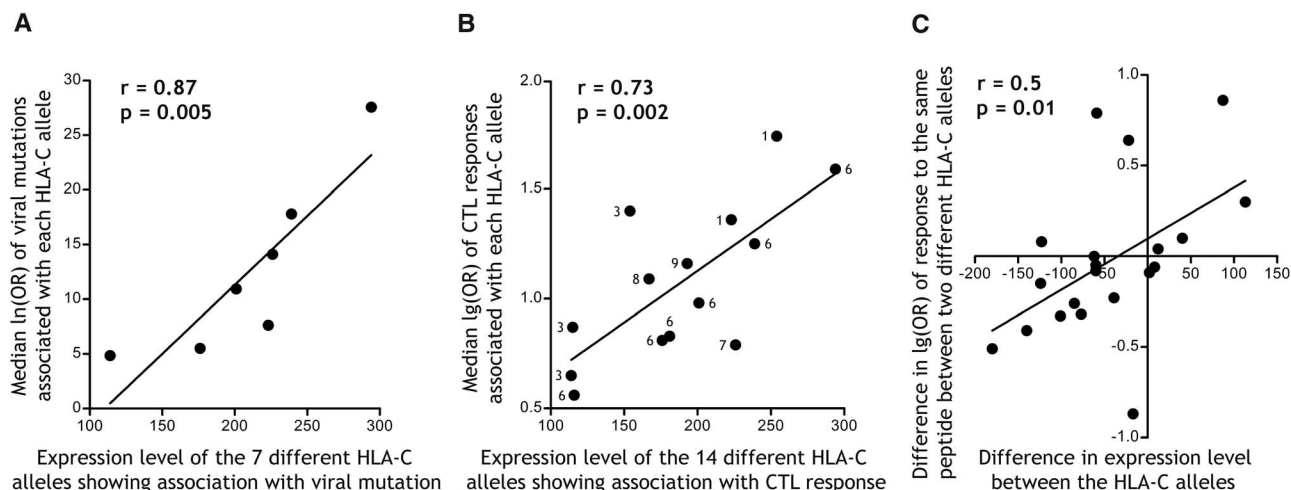
| Covariate               | <i>P</i>                             | HR          | 95% CI      |             |
|-------------------------|--------------------------------------|-------------|-------------|-------------|
| <b>HLA-C expression</b> | <b><math>1 \times 10^{-6}</math></b> | <b>0.67</b> | <b>0.55</b> | <b>0.74</b> |
| B*57:01                 | $2 \times 10^{-5}$                   | 0.30        | 0.17        | 0.52        |
| C*06:02                 | $7 \times 10^{-4}$                   | 1.75        | 1.26        | 2.41        |
| A*26:01                 | $2 \times 10^{-3}$                   | 0.43        | 0.25        | 0.73        |
| B*35:03                 | $2 \times 10^{-3}$                   | 2.11        | 1.3         | 3.41        |
| A*11:01                 | $3 \times 10^{-3}$                   | 0.62        | 0.45        | 0.85        |
| A*74                    | $4 \times 10^{-3}$                   | 0.26        | 0.11        | 0.65        |
| A*32:01                 | $4 \times 10^{-3}$                   | 0.53        | 0.34        | 0.82        |
| A*31:01                 | $3 \times 10^{-2}$                   | 0.56        | 0.33        | 0.95        |
| B*15:01                 | $4 \times 10^{-2}$                   | 0.72        | 0.52        | 0.99        |
| C*04:01                 | $4 \times 10^{-2}$                   | 1.30        | 1.01        | 1.69        |

HLA-C expression level for allotype pairs and the difference in strength of association with CTL response to the same peptide when restricted by these two allotypes ( $r = 0.50$ ,  $P = 0.01$ ) (Fig. 2C). That is, the likelihood of initiating a CTL response to a given peptide correlated positively with a greater increase in expression between the two alleles compared. These results suggest that the protective mechanism conferred by increased HLA-C expression level occurs in part

through an enhanced CTL-mediated immune response.

The effect of HLA-C expression level in HIV disease raises the possibility of its involvement in the risk of other human diseases. We tested this possibility in case-control cohorts of two inflammatory bowel diseases (IBD): Crohn’s disease (CD) and ulcerative colitis (UC). HLA-C expression as a continuous variable was considered in stepwise selection models along with all HLA





**Fig. 2. HLA-C expression level correlates with frequency of viral escape mutation and HIV-specific CTL responses.** (A) Analysis of viral sequences from 1888 Clade B-infected individuals revealed 12 epitopes containing viral mutations that were associated independently with an HLA-C allele. Median ln(OR) for associations with each of the seven different HLA-C alleles involved correlated positively with the expression level of these alleles. (B) Clade C-infected Africans ( $n = 1010$ ) were screened for CTL responses to overlapping peptides spanning the HIV proteome, and median

log OR of all responses independently associated with each HLA-C allele are plotted. The number of peptide responses associated with each HLA-C allele is labeled next to each point (total number of CTL responses is 71). At least one HLA-C-restricted CTL response was detected in 71% of individuals in the population. (C) Differences in the odds of detecting a response to the same peptide when restricted by different HLA-C alleles correlate with the difference in expression level of these allelic pairs. Pearson coefficients are reported for each correlation.

class I and II alleles with phenotypic frequencies of  $\geq 2\%$ . We include class II alleles because they show the most consistent allelic effects and the strongest MHC signal in GWAS of IBD (16–18). HLA-C expression level showed the second most significant effect in CD ( $P = 3 \times 10^{-7}$ , OR = 1.35 for an increase of 100 MFI units) after the well-established risk allele DRB1\*01:03 (19) (table S6). HLA-C expression level showed no effect in UC, although previously defined risk factors, such as HLA-DRB1\*01:03, were observed (16) ( $P = 1 \times 10^{-12}$ , OR = 2.03) (table S6). A greater role of Th1 response in CD relative to UC may be related to the influence of HLA-C expression level in risk of CD but not UC (20).

It is a novel facet of human genetic variation that HLA class I alleles vary in their expression level between normal individuals to an extent that modulates the efficacy of an immune response. The importance of HLA expression level is well appreciated, however, because one of the most striking cellular changes in the inflammatory response is interferon- $\gamma$  mediated up-regulation of HLA expression (21). As few as three peptide-MHC complexes can suffice for CTL killing in vitro (22), with increasing density improving efficiency of cytotoxicity and modulating the response to include secretion of inflammatory cytokines (23). Given the vast range of peptides bound by HLA class I allotypes, many individual peptides are likely present below the threshold required for CTL activation or at a level where increasing their density would influence the response (24). Thus, differences in expression of even twofold, which have been shown to improve the efficacy of CTL responses in vivo (25), could result in more beneficial control of HIV by more highly expressed HLA-C allotypes.

Unlike its protection in HIV infection, high expression increases the risk of CD. Contrasting effects of HLA-C expression level across human diseases may have contributed to the evolutionary history of the HLA-C locus, where expansion of higher expression HLA-C allotypes has occurred over the past 4 to 5 million years since their genesis, while maintaining lower expression allotypes (26). Thus, the complexity of HLA effects in disease pathogenesis go beyond peptide specificity to include the strength of immune responses as dictated by levels of HLA expression.

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12568), Swiss HIV Cohort Study (supported by the Swiss National Science Foundation grant number 33CSO-108787), Study on the Consequences of Protease Inhibitor Era (supported by grants R01 AI087145, K24AI069994, P30 AI027763, UL1 RR024131, P30 MH62246, and R24 AI067039), and MACS cohorts (funded by NIAID, with supplemental funding from the National Cancer Institute and the National Heart, Lung, and Blood Institute [grants U01-AI-35042, 5-MO1-RR-00722 (GCRC), U01-AI-35043, U01-AI-37984, U01-AI-35039, U01-AI-35040, U01-AI-37613, and U01-AI-35041]).

## Supplementary Materials

www.sciencemag.org/cgi/content/full/340/6128/87/DC1  
Materials and Methods  
Figs. S1 and S2  
Tables S1 to S7  
References (27–40)

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# Transposition-Driven Genomic Heterogeneity in the *Drosophila* Brain

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Recent studies in mammals have documented the neural expression and mobility of retrotransposons and have suggested that neural genomes are diverse mosaics. We found that transposition occurs among memory-relevant neurons in the *Drosophila* brain. Cell type-specific gene expression profiling revealed that transposon expression is more abundant in mushroom body (MB)  $\alpha\beta$  neurons than in neighboring MB neurons. The Piwi-interacting RNA (piRNA) proteins Aubergine and Argonaute 3, known to suppress transposons in the fly germline, are expressed in the brain and appear less abundant in  $\alpha\beta$  MB neurons. Loss of piRNA proteins correlates with elevated transposon expression in the brain. Paired-end deep sequencing identified more than 200 de novo transposon insertions in  $\alpha\beta$  neurons, including insertions into memory-relevant loci. Our observations indicate that genomic heterogeneity is a conserved feature of the brain.

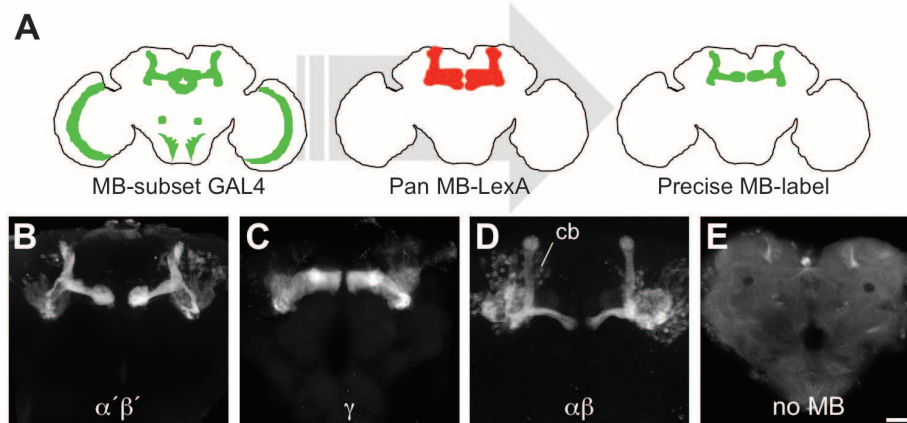
Transposons constitute nearly 45% of the human genome and 15 to 20% of the fly genome (1–3). Mobilized transposons can act as insertional mutagens and create lesions where they once resided (2). Recombination between homologous transposons can also delete intervening loci. Specific regions of the mammalian brain, such as the hippocampus, might be particularly predisposed to transposition (4, 5). LINE-1 (L1) retrotransposons mobilized during differentiation appear to insert in the open chromatin of neurally expressed genes (4–6). One such insertion in neural progenitor cells altered the expression of the receiving gene and the subsequent maturation of these cells into neurons (6). The mosaic nature of transposition could therefore provide additional neural diversity that might contribute to behavioral individuality and/or neurological disorders (7–9).

The *Drosophila melanogaster* mushroom bodies (MBs) are brain structures critical for olfactory memory. The approximately 2000 intrinsic MB neurons are divisible into  $\alpha'\beta'$ ,  $\gamma$ , and  $\alpha\beta$

according to their morphology and roles in memory processing (10–13). Here, we used cell type-specific gene expression profiling (14) to gain insight into cellular properties of MB neurons. Intersectional genetics (15) (Fig. 1A) allowed us to exclusively label MB  $\alpha'\beta'$ ,  $\gamma$ , and  $\alpha\beta$  neurons in the brain with green fluorescent protein (GFP) (Fig. 1, B to D). For comparison, we also assayed a “no MB” genotype in which GFP labels other neurons in the brain but not MB neurons (Fig. 1E). Sixty brains per genotype were dissected from the head capsule and dissociated by proteolysis and agitation; GFP-expressing single cell bodies

were then collected by fluorescence-activated cell sorting (FACS). Total RNA was isolated from 10,000 cells per genotype, and polyadenylated RNA was amplified and hybridized to Affymetrix *Drosophila* 2.0 genome expression arrays. Each genotype was processed in four independent replicates (Fig. 2A).

Routine statistical analysis for differentially expressed genes, including a multiple-testing correction across all 16 data sets, did not reveal significant differences at a false discovery rate (FDR) of  $<0.05$ . We therefore used CARMAweb (16) to identify 146 mRNAs whose average signal was  $\geq 7$  in  $\alpha\beta$  neurons and that were also higher than in  $\alpha'\beta'$  neurons by a factor of  $\geq 2$ . Of the top 60 transcripts from this list, 29 were significantly different from  $\alpha'\beta'$  signals (by raw  $P$  value) and represent transposons (Fig. 2A and table S1). Alignment of the corresponding values from the  $\gamma$  and no-MB profiles showed a similarly significant bias in transposon expression over these samples. We identified retrotransposons that transpose via a replicative mechanism involving an RNA intermediate and DNA elements that use nonreplicative excision and repair. Retrotransposons can be subdivided into long-terminal repeat (LTR) elements and long interspersed nuclear elements (LINEs). We found 11 LTR elements (*Tabor*, *mdg1*, *roo*, *qbert*, *gypsy*, *invader3*, *gypsy2*, *microcopia*, *412*, *accord*, and *blood*), 11 LINE-like elements (*G6*, *RT1b*, *HeT-A*, *Ivk*, *Cr1a*, *F* element, *Doc2*,



**Fig. 1. Exclusive labeling of MB neuron subsets in the fly brain.** (A) MB-expressing GAL4 transgenic fly strains with expression elsewhere in the brain were intersected with a pan MB-expressing LexA. See supplementary materials for details of genetic approach. (B to E) Confocal projection of individual brains from flies, labeling  $\alpha'\beta'$  neurons (B),  $\gamma$  neurons (C),  $\alpha\beta$  neurons (D), and all neurons in the brain except MB neurons (E). Scale bar, 40  $\mu$ m.

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*baggins*, *R2*, *Doc3*, and *Doc*), and four DNA elements (*Bari1*, *pogo*, *Tc3*, and *transib3*).

We further analyzed 14 transposons, representing the most abundant in each class. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) of RNA from independently purified cell samples confirmed that transposon expression was significantly higher in  $\alpha\beta$  neurons than in other MB neurons (Fig. 2B). All transposons, other than *R2*, were also significantly higher in  $\alpha\beta$  neurons than in the rest of the brain. *R2* is unique, because it exclusively inserts in the highly repeated 28S rRNA locus and heterochromatin (17).

Transposition is ordinarily regulated by chromatin structure and posttranscriptional degradation of transposon mRNA guided by complementary RNAs (18–25). The small interfering RNA (siRNA) pathway has been implicated in somatic cells (26). In contrast, the Piwi-interacting RNA (piRNA) pathway has a more established role in the germline (19–21). The microarray analysis skewed our attention toward piRNA because the expression level of the translocated *Stellate* locus, *Stellate12D orphon* (*Ste12DOR*) mRNA, was higher in  $\alpha\beta$  than in other MB neurons and the rest of the brain by a factor of  $>20$  (Fig. 2A and table S1). *Stellate* repeat transcripts are usually curtailed by piRNA, not siRNA (27, 28). *Stellate* repeats encode a casein kinase II regulatory subunit, and piRNA mutant flies form *Stellate* protein crystals in testis (29). Immunostaining *Stellate* in the brain labeled puncta within  $\alpha\beta$  dendrites in the MB calyx, consistent with high *Ste12DOR* expression in wild-type  $\alpha\beta$  neurons (fig. S1).

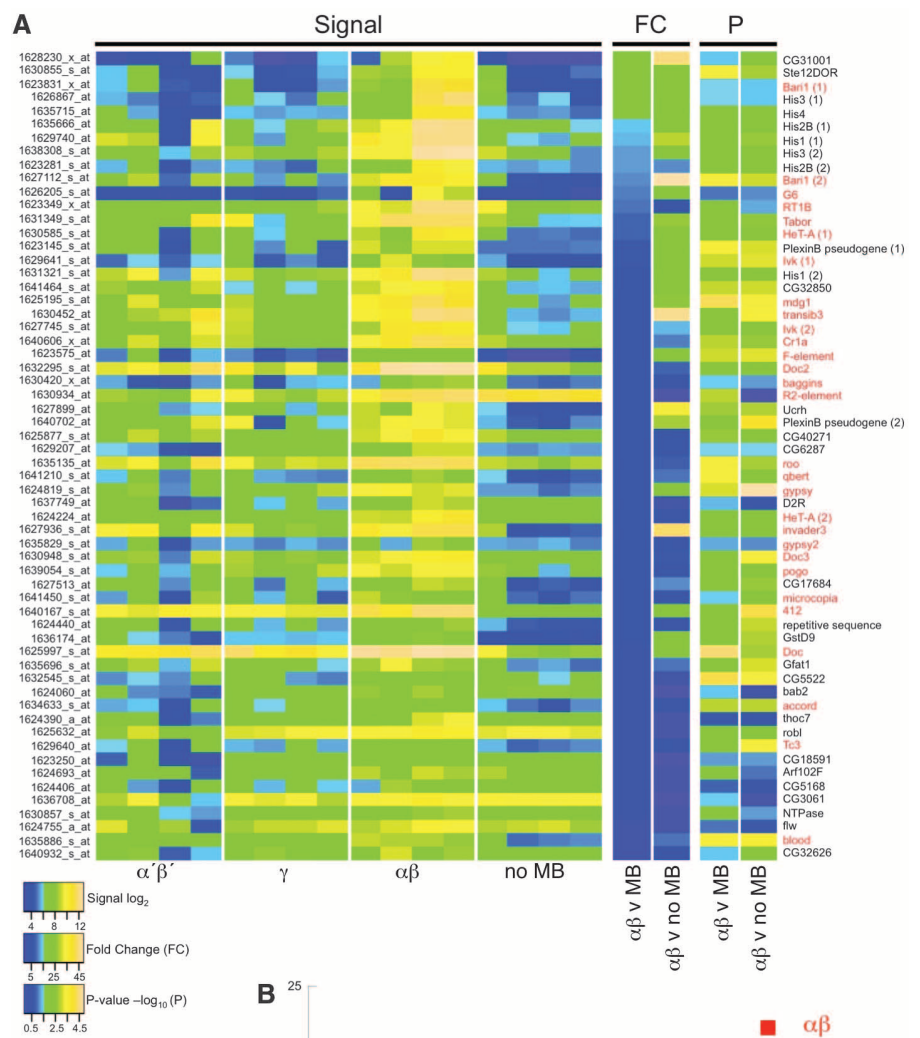
piRNAs are loaded into the Piwi clade argonaute proteins Piwi, Aubergine (Aub), and Argonaute 3 (Ago3) (21). Piwi and Aub can amplify piRNA pools with Ago3 (30, 31). To investigate piRNA involvement in differential transposon expression, we immunolocalized Piwi proteins and colocalized GFP to assign signals to MB neuron type (Fig. 3). Aub and Ago3 differentially labeled MB subdivisions in addition to structures throughout the brain (Fig. 3 and figs. S2 and S3), but we did not detect Piwi (32). The ellipsoid body of the central complex stained strongly for Aub but not at all for Ago3 (Fig. 3, A and E, and figs. S2D and S3D), which suggests possible functional exclusivity of Piwi proteins in the brain.

Differential Aub and Ago3 labeling was most evident within axon bundles in the peduncle and lobes, where MB neuron types are anatomically discrete (Fig. 3, A to H, and fig. S2). Aub protein colocalized with  $\gamma$  and  $\alpha'\beta'$  neurons in the peduncle and lobes but was reduced in  $\alpha\beta$  neurons in both locations (Fig. 3, B to D, and fig. S2). Ago3 did not label MB lobes (fig. S3) but colocalized with  $\gamma$  neurons in the peduncle (Fig. 3H). Ago3 labeled core  $\alpha\beta$  ( $\alpha\beta_c$ ) neurons but did not label outer  $\alpha\beta$  neurons (Fig. 3F). Therefore, outer  $\alpha\beta$  neurons do not abundantly express Aub or Ago3, which implies that transposon suppression is relaxed. In contrast,  $\gamma$  neurons

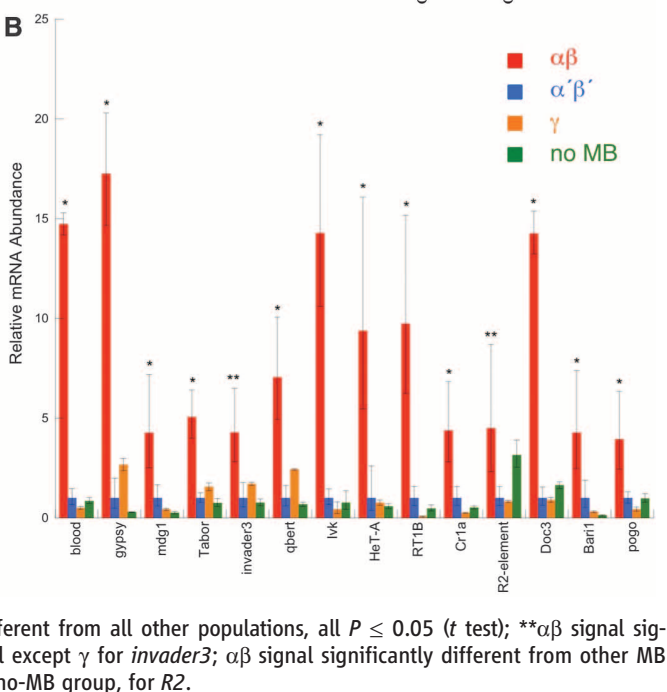
express Aub and Ago3, providing potential for piRNA amplification, and  $\alpha'\beta'$  neurons express Aub. These patterns of Aub and Ago3 in the MB peduncle appear conserved in brains from

*D. erecta*, *D. sechellia*, and the more distantly related *D. pseudoobscura* species (fig. S4).

Loss of siRNA function elevates transposon expression in the head (22). We replicated these



**Fig. 2. Gene expression profiling of MB neurons.** (A) Microarray data emphasizing elevated expression in  $\alpha\beta$  neurons. Each signal column per category represents an independent replicate. Fold change (FC) and *t*-test *P* values are shown for  $\alpha\beta$  versus other MB neurons (average value for  $\alpha'\beta'$  and  $\gamma$ ) and  $\alpha\beta$  versus the no-MB group. Transposons are denoted in red. Color scale bars are linear for FC,  $-\log_{10}$  for *P* value, and  $\log_2$  for signal level. (B) qRT-PCR validation of increased transposon mRNA levels in  $\alpha\beta$  neurons. \* $\alpha\beta$  signal significantly different from all other populations, all  $P \leq 0.05$  (*t* test); \*\* $\alpha\beta$  signal significantly different from all except  $\gamma$  for *invader3*;  $\alpha\beta$  signal significantly different from other MB neurons, but not from the no-MB group, for *R2*.





findings with *ago*<sup>2414</sup> and *dcr-2*<sup>L8116X</sup> mutant flies (fig. S5). In parallel, we used trans-heterozygous *aub* (*aub*<sup>HN2</sup>/*aub*<sup>OC42</sup>) and *ago3* heads (*ago3*<sup>12</sup>/*ago3*<sup>13</sup>) (19, 33) and trans-heterozygous *armitage* heads (*armi*<sup>1</sup>/*armi*<sup>72.1</sup>) (34) to test whether piRNA suppressed transposon expression (Fig. 3I). Levels of the 14 LTR, LINE-like, and TIR group transposons verified to be expressed in  $\alpha\beta$  neurons (Fig. 2B) were assayed by qRT-PCR; of these 14 transposons, 13 were significantly elevated in siRNA-defective *ago2* and *dcr-2* mutants (fig. S5). The piRNA-defective *aub*, *ago3*, and *armi* mutants also exhibited significantly elevated levels of 9 of the 14 elements (Fig. 3I). Levels of the LTR elements *gypsy*, *Tabor*, and *qbert*; the LINE-like elements *HeT-A*, *RT1b*, and *R2*; and the TIR element *pogo* were higher in *ago3* mutants. In addition, *blood*, *Tabor*, and *R2* were elevated in *aub* mutants, and *blood*, *gypsy*, *Tabor*, *invader3*, *qbert*, *HeT-A*, and *R2* were elevated in *armi* mutants. Therefore, the piRNA pathway contributes to transposon silencing in the brain, and low levels of *Aub* and *Ago3* may permit expression in  $\alpha\beta$  neurons.

To determine whether transposons are mobile, we mapped new insertions by deep sequencing (35) of  $\alpha\beta$  DNA (Fig. 4). We purified  $\alpha\beta$  neurons by FACS, as for transcriptome analysis, but isolated genomic DNA. Insertions were defined by paired-end reads in which one end mapped to the annotated genome and the other to the transposon

sequence. To identify de novo transposition events in  $\alpha\beta$  neurons, we compared the genomic position of transposons within the  $\alpha\beta$  sequence to those located by sequencing DNA from genetically identical embryos. In addition, we sequenced DNA from the remainder of the brain tissue from the FACS separation of  $\alpha\beta$  neurons (Fig. 4A and table S4).

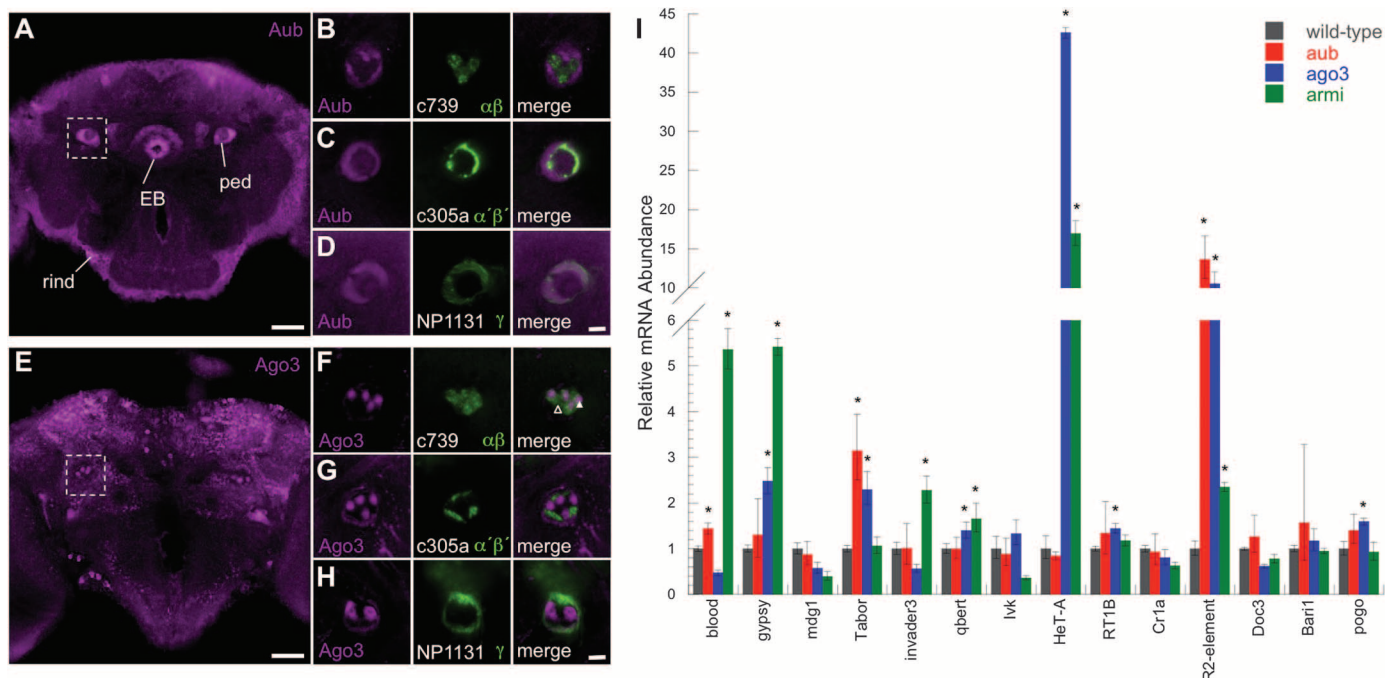
These studies identified 3890 transposon insertions in embryo DNA that differed from the published *Drosophila* genome sequence (Fig. 4A). In comparison,  $\alpha\beta$  neuron DNA revealed 215 additional sites. The remaining brain tissue uncovered 200 new insertions, including 19 that were identical to those in  $\alpha\beta$  neurons. The sequencing depth for embryos (34.1 $\times$ ) was an order of magnitude greater than for neurons (3.1 $\times$ ) because embryo material could be collected more easily; hence, the  $\alpha\beta$  and other brain insertions are likely de novo. By randomly sampling reads to yield 1 $\times$  genome coverage, we calculated 129 new transposon insertions per  $\alpha\beta$  neuron genome (table S2). Sequencing single neurons (36) would reveal the exact cellular frequency and heterogeneity of transposition events.

New  $\alpha\beta$  insertions occurred across all chromosomes, without obvious regional bias (Fig. 4B). In addition, insertions resulted from 49 different transposons representing LTR, LINE-like, TIR, and *Foldback* (FB) classes (Fig. 4C). They included 11 of the 29 transposons in the

$\alpha\beta$  transcriptome (Fig. 2A), and the number of insertions per class was consistent with their prevalence in the genome (Fig. 4D). Therefore, many transposons mobilize in  $\alpha\beta$  neurons.

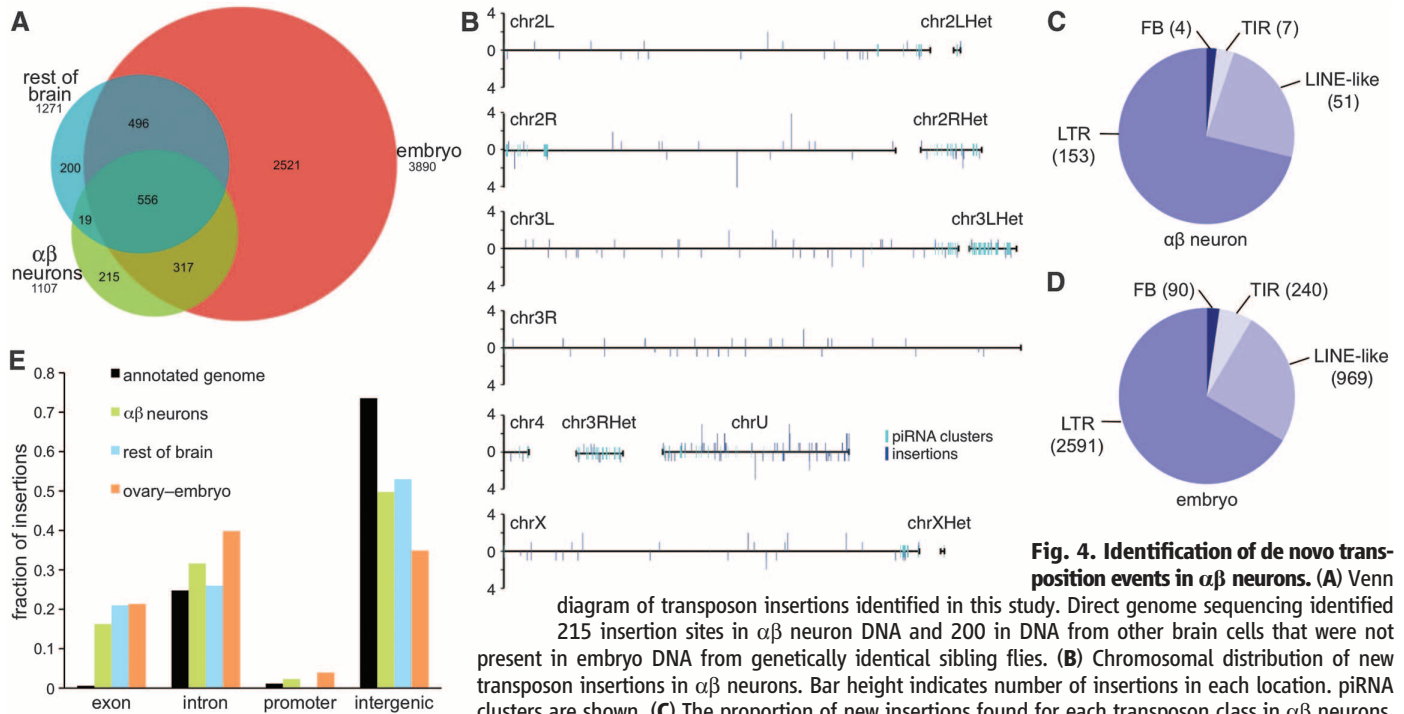
Of the 215 de novo  $\alpha\beta$  insertions, 108 mapped close to identified genes (Fig. 4E and fig. S6). Of these, 35 disrupted exons, 68 disrupted introns, and 5 fell in promoter regions (<1 kb from transcription start site). The remaining 107 insertions mapped to piRNA clusters or intergenic regions and were not assigned to a particular gene. A similar distribution was observed for the 200 new insertions in the rest of the brain (Fig. 4E and fig. S6). The reference fly genome has 258 transposon insertions in exonic regions, 11,110 insertions in intronic regions, 502 insertions in promoter regions, and 33,008 insertions in intergenic regions. Therefore, both groups of brain cells had a significantly larger fraction of insertions within exons, and fewer in intergenic regions, than the transposons that are annotated in the genome (Fig. 4E and table S8). To test whether such a distribution was unique to neurons, we analyzed de novo insertions in ovary DNA, again using embryo sequence as the comparison. New insertions in ovary DNA revealed a similar skew toward exons (Fig. 4E and table S8).

In mammals, active L1 elements appear to disrupt neurally expressed genes (4–6). New  $\alpha\beta$  neuron insertions, but not those in other tissue



**Fig. 3. *Aub* and *Ago3* are not abundant in  $\alpha\beta$  neurons.** (A) *Aub* immunostaining (magenta) labels the ellipsoid body (EB), MB subdivision in the peduncle (ped), and cell body layer (rind). A single confocal section is shown at the level of the MB peduncle. Dashed box denotes area shown in (B) to (D). (B to D) *Aub* labels  $\alpha\beta'$  and  $\gamma$  neurons in the peduncle more than  $\alpha\beta$  neurons. *Aub* staining (B) is mutually exclusive to GFP-expressing  $\alpha\beta$  neurons (green) but overlaps with  $\alpha\beta'$  neurons (C) and  $\gamma$  neurons (D). (E) *Ago3* immunostaining (magenta) labels neurons throughout the brain and MB subdivision in the

peduncle. A single confocal section is shown at the level of the MB peduncle. Dashed box denotes area shown in (F) to (H). (F to H) *Ago3* staining is prominent in the  $\alpha\beta$  core [(F), solid triangle] and does not overlap with outer GFP-labeled  $\alpha\beta$  neurons [(F), open triangle], nor with  $\alpha\beta'$  neurons (G), but overlaps with  $\gamma$  neurons (H). Scale bars, 40  $\mu$ m [(A) and (E)], 10  $\mu$ m [(B) to (D), (F) to (H)]. (I) qRT-PCR analysis from wild-type, *aub*<sup>HN2</sup>/*aub*<sup>OC42</sup>, *ago3*<sup>12</sup>/*ago3*<sup>13</sup>, and *armi*<sup>1</sup>/*armi*<sup>72.1</sup> mutant fly brains. Several transposon transcripts are elevated in *ago3*, *aub*, and *armi* mutant fly brains. Values are normalized to wild-type heads. \*Significant increase,  $P < 0.05$  (t test).



**Fig. 4. Identification of de novo transposition events in  $\alpha\beta$  neurons.** (A) Venn

diagram of transposon insertions identified in this study. Direct genome sequencing identified 215 insertion sites in  $\alpha\beta$  neuron DNA and 200 in DNA from other brain cells that were not present in embryo DNA from genetically identical sibling flies. (B) Chromosomal distribution of new transposon insertions in  $\alpha\beta$  neurons. Bar height indicates number of insertions in each location. piRNA clusters are shown. (C) The proportion of new insertions found for each transposon class in  $\alpha\beta$  neurons. (D) Proportion of insertions per transposon class in the inherited fly genome from our sequencing of

embryo DNA. (E) Distribution of transposons in the annotated genome significantly differs from distribution of de novo insertions in  $\alpha\beta$  neurons, the rest of the brain, and ovary DNA with respect to neighboring genes ( $P < 2.2 \times 10^{-16}$  for  $\alpha\beta$  neurons, the rest of the brain, and ovary;  $\chi^2$  test).

(tables S7 and S9), were significantly enriched in 12 Gene Ontology (GO) terms (Benjamini FDR  $< 5\%$ ; tables S4 and S5), all of which are related to neural functions. Moreover, promoter regions from 18 of 20 of the targeted genes drive expression in  $\alpha\beta$  neurons (table S10). We found exonic insertions in *gilgamesh*, *derailed*, and *mushroom body defect* and intronic insertions in *dunce* and *rutabaga* (table S3), all of which have established roles in MB development and function (37–40). In addition, MB neurons are principally driven by cholinergic olfactory projection neurons (41) and receive broad GABA-ergic inhibition (42) and dopaminergic modulation through G protein-coupled receptors (43). We identified intronic insertions in *nicotinic Acetylcholine Receptor  $\alpha$  80B*, *G protein-coupled receptor kinase 1*, and *cyclic nucleotide gated channel-like* and an exonic insertion in *GABA-B-receptor subtype 1* (table S3). Transposon-induced mosaicism could therefore alter integrative and plastic properties of individual MB  $\alpha\beta$  neurons.

Our data establish that transposon-mediated genomic heterogeneity is a feature of the fly brain and possibly other tissues. Together with prior work in rodents and humans (4–6), our results suggest that genetic mosaicism may be a conserved characteristic of certain neurons. Work in mammals indicates that L1 expression occurs because the L1 promoter is released during neurogenesis (6, 21). Our data are consistent with such a model and also support the idea that transposons avoid posttranscriptional piRNA silencing in adult  $\alpha\beta$  neurons.

A recent study described a role for piRNA in epigenetic control of memory-related gene expression in *Aplysia* neurons (44). It is therefore possible that MB neurons differentially use piRNA to control memory-relevant gene expression and that transposon mobilization is an associated cost. Because we found transposon expression in  $\alpha\beta$  neurons of adult flies, it is conceivable that disruptive insertions accumulate throughout life, leading to neural decline and cognitive dysfunction. Alternatively, permitting transposition may confer unique properties across the 1000 neurons in the  $\alpha\beta$  ensemble and potentially produce behavioral variability between individual flies in the population.

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nlm.nih.gov/sites/sra) archives as E-MEXP-3798 and SRP017718, respectively. We thank A. Vodala, J. Menet, K. Abruzzi, and N. Francis for advice with techniques. Supported by a Wellcome Trust Senior Research Fellowship in Basic Biomedical Sciences, the Gatsby Charitable Foundation, Oxford Martin School and grants MH069883 and MH081982 from NIH (S.W.), NIH grants

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### Supplementary Materials

www.sciencemag.org/cgi/content/full/340/6128/91/DC1  
Materials and Methods

Figs. S1 to S6  
Tables S1 to S10  
References (45–49)

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# Rats and Humans Can Optimally Accumulate Evidence for Decision-Making

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The gradual and noisy accumulation of evidence is a fundamental component of decision-making, with noise playing a key role as the source of variability and errors. However, the origins of this noise have never been determined. We developed decision-making tasks in which sensory evidence is delivered in randomly timed pulses, and analyzed the resulting data with models that use the richly detailed information of each trial's pulse timing to distinguish between different decision-making mechanisms. This analysis allowed measurement of the magnitude of noise in the accumulator's memory, separately from noise associated with incoming sensory evidence. In our tasks, the accumulator's memory was noiseless, for both rats and humans. In contrast, the addition of new sensory evidence was the primary source of variability. We suggest our task and modeling approach as a powerful method for revealing internal properties of decision-making processes.

Decisions in real life often need to be made based on noisy or unreliable evidence. Accumulating evidence from a set of noisy observations made over time makes it possible to average over different noise samples, thus improving estimates of the underlying signal. This principle is the basis for the influential class

of “drift-diffusion” models (1–5), which have been broadly applied to explain a variety of phenomena in biology (6–8). Accumulation involves both maintaining a memory of evidence accrued so far and adding new evidence to the memory. Yet no test to date has distinguished between noise associated with each of these two components.

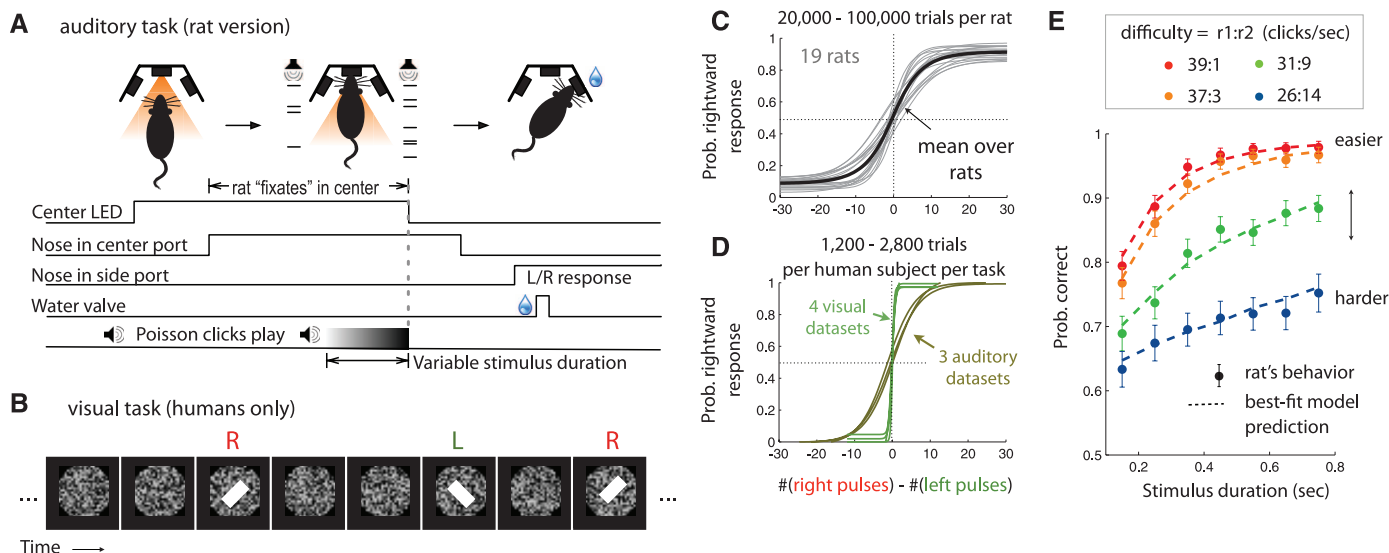
We developed tasks in which subjects (humans and rats) were concurrently presented with two trains of pulses, one train representing “left”-labeled pulses and the other, “right”-labeled pulses. At the end of each trial, the subjects had to report which of the two trains had the greater total number of pulses. The timing of pulses was random and varied widely, both within and across individual trials (9, 10). We reasoned that the precisely known pulse timing would enable detailed modeling of the subjects' choices on each individual trial, whereas its variability would allow exploration of the stimulus space and would thus provide statistical power.

In an auditory version of the task, performed by three humans and 19 rats, left pulse trains were clicks presented on a speaker to the left of the subject, and right pulse trains were clicks presented on a speaker to the right of the sub-

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**Fig. 1. Psychophysical tasks and summary of behavior.** (A) Sequence of events in each trial of the rat auditory task. After light onset from a light-emitting diode (LED) in a center port, trained rats placed their nose into the port and “fixated” their nose there for a fixed amount of time until the light was turned off (1 to 2 s). Trains of randomly timed clicks were played concurrently from left and right free-field speakers during the last portion of the fixation time. After nose fixation and sounds ended, the rat made a choice, poking in the left or the right port to indicate which side played more clicks. Humans performed an analogous version of the task on a computer while

wearing headphones. (B) Schematic diagram of a stimulus in the visual pulses version of the task, performed by humans on a computer. (C) Psychometric curves (fits to a four-parameter logistic function for each subject; see methods) for rat subjects. (D) Psychometric curves, as in (C), for human subjects. (E) Chronometric curves for an example rat. Difficulty is labeled by the ratio of click rates played on the two sides. For each difficulty, performance improves with longer stimulus durations. Dashed lines show the best-fit model predictions for this rat, as described in the text. The vertical axis shows mean accuracy and 95% confidence interval (CI).



ject (Fig. 1A; free-field speakers for rats, headphones for humans). In a visual version of the task, performed by four humans, left pulses were flashed white bars, tilted anticlockwise from the vertical, and right pulses were flashed white bars tilted clockwise (3) (Fig. 1B). On each trial, the stimulus was presented for a duration controlled by the experimenter. The sum of the two pulse rates was kept fixed within each task, and discrimination difficulty was controlled on each trial by the ratio of the two rates (Fig. 1, C to E).

To examine a large variety of possible mechanisms consistent with the performance improvement at longer stimulus durations seen in Fig. 1E [(11, 12); see also fig. S4], we expanded on the drift-diffusion framework and implemented a flexible model (Box 1) in which different regimes of model parameter values represent widely different mechanisms (three examples in Fig. 2A), with mixtures of mechanisms represented by intermediate parameter values.

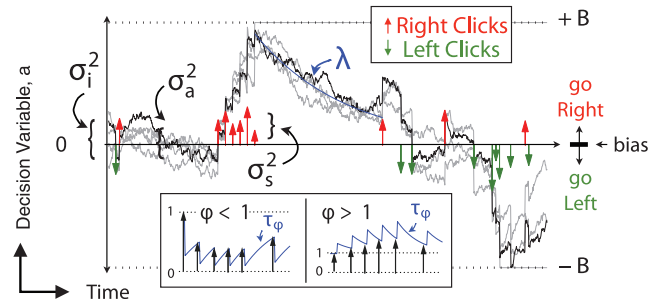
Given a trial's specific pulse times and a set of parameter values, the model produces the probability of observing a left versus a right response on that trial. Methods to compute the gradient of this probability with respect to model parameters (see the supplementary materials) were critical for efficiently finding the parameter values that gave the maximum likelihood of observing the complete set of a subject's responses. Numerical tests always found only one maximum (fig. S6), suggesting that we always found the global maximum. Consistent with this observation, a mathematically related model has been proven to have a concave log likelihood (6), suggesting that our model may also be provably concave and have a single maximum.

Figure 2, B to D, shows the likelihood landscape around best-fit (i.e., maximum-likelihood) parameters, given the data of a representative rat subject. Confidence intervals are given by the parameter width of the maximum (blue contours). Figure 2B shows  $\lambda$  ( $= 1/\tau$ , the memory time constant), which represents accumulator memory leak (if  $\lambda < 0$ ) or instability (if  $\lambda > 0$ ), and  $B$ , the height of the decision-commitment evidence bounds.  $\lambda$  was statistically indistinguishable from zero. That is, the decision dynamics were neither leaky nor unstable, suggesting that sensory evidence from throughout the entire stimulus period was given equal weight. The best-fit  $B$  was large enough that it produced model fits indistinguishable from those produced by  $B = \infty$ . Across subjects (Fig. 2, E and H), species, sensory modalities, and task parameters, the accumulator's memory time constant  $|\tau| = 1/|\lambda|$  was long ( $|\lambda| = 0.91 \pm 0.15 \text{ s}^{-1}$  mean  $\pm$  SE across rats;  $|\lambda| = 0.23 \pm 0.071 \text{ s}^{-1}$  across humans), in the sense that  $|\tau|$  was comparable to or greater than the longest stimulus duration used [1 s for rats, 4 s for humans (13)]. The best-fit values of  $B$  and  $\lambda$  were thus in the gradual accumulation regime (Fig. 2A, top).

In our tasks, noise in the sensory evidence ( $\sigma_s^2$ ) adds total variance proportional to the sum

of the amplitude of the clicks, whereas the memory diffusion noise ( $\sigma_a^2$ ) adds total variance proportional to the stimulus duration. This separability allows us to isolate the magnitude of the diffusion noise  $\sigma_a^2$  that gives the drift-diffusion model its name (2, 5). To our surprise, in 13 out of 19 rats and in all three humans performing the auditory task and all four humans performing the visual task, the value that best fit the data was the ideal  $\sigma_a^2 = 0$  (Fig. 2C for an example subject, Fig. 2, F and I, for all subjects). Consistent with the easily

distinguishable right versus left pulses used with humans, the best-fitting values of sensory evidence noise  $\sigma_s^2$  for humans were substantially lower than those for the rats (Fig. 2I). Again in this much lower  $\sigma_s^2$  regime, the best-fitting memory diffusion noise  $\sigma_a^2$  was zero. The dominant source of variability was thus noise in the evidence associated with each incoming pulse ( $\sigma_s^2 = 1.90 \pm 0.28 \text{ pulses}^2$  per incoming pulse for rats,  $0.50 \pm 0.059 \text{ pulses}^2$  in the human auditory task, and  $0.24 \pm 0.10 \text{ pulses}^2$  in the human visual task.)



### Box 1.

At each time point, the accumulator memory  $a$  (black trace) represents an estimate of the right versus left evidence accrued so far. At stimulus end, the model decides right if  $a > \text{bias}$  and left otherwise, where bias is a free parameter. The light gray traces indicate alternate runs with different instantiations of model noise.

Right  $\uparrow$  (left  $\downarrow$ ) pulses change the value of  $a$  by positive (negative) impulses of magnitude  $C$ .

$\sigma_i^2$  parameterizes noise in the the initial value of  $a$ .

$\sigma_a^2$  is a diffusion constant, parameterizing noise in  $a$ .

$\sigma_s^2$  parameterizes noise when adding the evidence from a right or left pulse: For each click, variance  $\sigma_s^2$  is scaled by the amplitude of  $C$  and then added to the evidence contributed by the click.

$\lambda$  parameterizes consistent drift in the memory  $a$ . In the "leaky" or forgetful case ( $\lambda < 0$ , illustrated), drift is toward  $a = 0$ , and later pulses affect the decision more than earlier pulses. In the "unstable" or impulsive case ( $\lambda > 0$ ), drift is away from  $a = 0$ , and earlier pulses affect the decision more than later pulses. The memory's time constant  $\tau = 1/\lambda$ .

$B$  is the height of the sticky decision bounds and parameterizes the amount of evidence necessary to commit to a decision.

$\phi$  and  $\tau_\phi$  parameterize sensory adaptation by defining the dynamics of  $C$ . Immediately after a click, the magnitude  $C$  is multiplied by  $\phi$ .  $C$  then recovers toward an unadapted value of 1 with time constant  $\tau_\phi$ . Facilitation is thus represented by  $\phi > 1$ , whereas depression is represented by  $\phi < 1$  (inset).

These properties are implemented by the following equations

if  $|a| \geq B$  then  $da/dt = 0$ ; else

$$da = \sigma_a dW + (\delta_{t,t_R} \cdot \eta_R \cdot C - \delta_{t,t_L} \cdot \eta_L \cdot C) dt + \lambda a dt \quad (1)$$

where  $\delta_{t,t_{R,L}}$  are delta functions at the times of the pulses;  $\eta$  are i.i.d. Gaussian variables drawn from  $N(1, \sigma_c)$ ; and  $dW$  is a white-noise Wiener process. The initial condition  $a(t=0)$  is drawn from the Gaussian  $N(0, \sigma_i)$ .

Adaptation dynamics are given by

$$\frac{dC}{dt} = \frac{1-C}{\tau_\phi} + (\phi - 1)C(\delta_{t,t_R} + \delta_{t,t_L}) \quad (2)$$

In addition, a lapse rate parameterizes the fraction of trials on which a random response is made. Ideal performance ( $a = \text{\#right clicks} - \text{\#left clicks}$ ) would be achieved by

$$\lambda = 0, B = \infty, \sigma_a^2 = \sigma_s^2 = \sigma_i^2 = 0, \phi = 1, \text{bias} = 0 \quad (3)$$

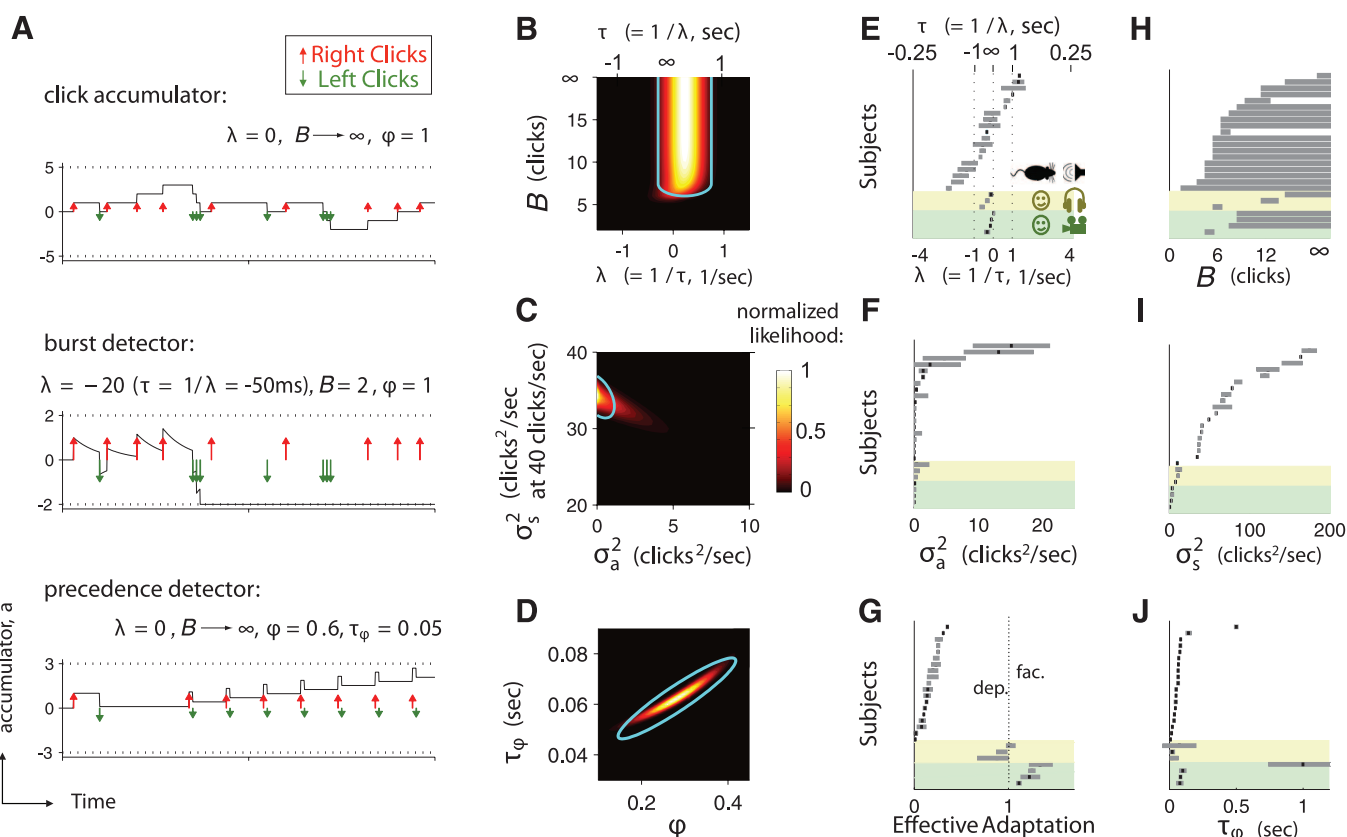
This variability could be introduced by sensory uncertainty in the left-versus-right classification of each individual pulse, or by noise in the process of adding new sensory evidence to the accumulator memory.

The pulsatile nature of our task made it straightforward to parameterize sensory adaptation [(14); Eq. 2]. We found strong, quickly-recovering depression for rats (Fig. 2, D, G, and J; adaptation magnitude  $\varphi = 0.17 \pm 0.021$ , recovery time constant  $\tau_\varphi = 0.080 \pm 0.024$  s, mean  $\pm$  SE across rats). This is consistent with the depression observed in auditory cortex neural responses to click train stimuli (15, 16). Stimuli in the human tasks were constructed with a minimum inter-pulse interval (30 ms in the auditory task, 150 ms in the visual task), and this greatly reduced the adaptation effects as compared to those in rats (Fig. 2, G and J). Across all adaptation regimes, we found long accumulator time constants and zero memory diffusion noise.

If our subjects' behavior depends on a process that cannot be approximated by the model [such as collapsing bounds (17), variability in attention (18), or other possibilities not yet formalized in a model], our interpretation of the best-fit values may be problematic. We therefore tested the model-derived conclusions. To assess the memory time constant  $\tau$ , we calculated the "psychophysical reverse correlation" (17, 19–21), which estimates the extent to which click rates at each point in time influence left and right decisions. This analysis indicated that all periods of the trial have similar influence on the decision (approximately constant separation between the two traces in Fig. 3A), which is consistent with the long  $\tau$  found in the model-based analysis. To assess our estimates of the single-pulse noise  $\sigma_s^2$  and the starting variability  $\sigma_a^2$ , we fit the model to data from trials with multiple clicks on each of the two sides, and used those best-fit parameters to predict performance on trials in

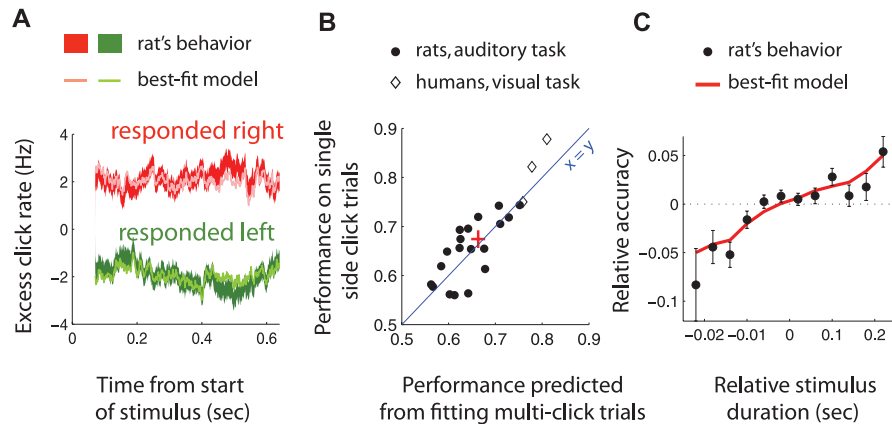
which only one single-side pulse happened to be presented (for which performance is dominated by  $\sigma_s^2$  and  $\sigma_a^2$ ). The prediction was accurate, even on an individual subject-by-subject basis (Fig. 3B). To assess the memory diffusion noise  $\sigma_a^2$ , we controlled for sensory evidence by dividing trials into groups, so that all trials within a group had the same number of right clicks and the same number of left clicks; assuming large  $|\tau|$ , performance within each group is then dominated by  $\sigma_a^2$  and the click depression parameters  $\varphi$  and  $\tau_\varphi$ . Large  $\sigma_a^2$  would predict decreasing within-group performance at longer stimulus durations. The data showed the opposite trend and was precisely predicted by the best-fit model, where  $\sigma_a^2 = 0$  and clicks are depressing (Fig. 3C and fig. S13). The tests of Fig. 3 thus provided model-independent confirmation of the model-fit parameter values.

Using highly variable yet precisely known stimuli, together with a trial-by-trial model that



**Fig. 2. The model can fit a variety of mechanisms, but the data consistently fit to a pulse-accumulating mechanism with zero noise in the accumulator's memory.** (A) Three examples of the mechanisms that the model can represent. Top: The ideal, a pulse accumulator that weights all pulses equally. Middle: A burst detector. If three or more pulses from the same side arrive within 50 ms, the sticky bounds are reached, meaning that a commitment to orient to that side is made. Bottom: A precedence detector. If pulses from one side tend to arrive shortly before pulses from the other side, the adaptation minimizes the second side's pulses, and the decision tends toward the preceding side. (B to D) Parameter-likelihood landscapes that result from the data of one example rat. Panels are two-dimensional slices, cut through the full nine-dimensional parameter space, around the best-fit values. The blue curves represent CIs [2 SD of the multidimensional normal distribution fit to the

likelihood landscape (28)]. The best-fit parameter values found, which are  $\lambda \approx 0$ ,  $B \gg 1$ ,  $\sigma_a^2 \approx 0$ ,  $\sigma_s^2 \gg 0$ , and  $\varphi \ll 1$ , correspond to pulse accumulation [(A), top] with a perfect memory but imperfect processing of sensory inputs. (E to J) Summaries of best-fit parameters over all subjects and tasks. Black ticks are best-fit values; gray bars span the CIs. Each panel has been divided by task (yellow highlight for human auditory task, green highlight for human visual task) and then sorted independently in order of parameter value. (E) All subjects, in all tasks, had long accumulator memories. (H) Most subjects were best fit with large bounds ( $B \rightarrow \infty$ ). (F) Thirteen of 19 rats and all humans in both auditory and visual tasks were best fit with  $\sigma_a^2 = 0$ . (I) A wide range of values, all large compared to  $\sigma_s^2$ , were found for  $\sigma_s^2$ . (G) and (J) All rats showed strong, rapidly recovering depression ( $\varphi < 1$ , mean  $\tau_\varphi = 0.040$  s). Humans showed weak depression in the auditory task and weak facilitation in the visual task.



**Fig. 3. Model-independent analyses support model-fitting results.** (A) Long  $\tau$ : psychophysical reverse correlation for an example rat (longest quarter of trials only). For each time point in each trial, we computed the excess pulse rate difference (right pulses/s – left pulses/s, relative to the value expected given the random processes used to generate the trial) and then obtained an average for trials resulting in a right (red) and an average for trials resulting in a left (green) decision. The separation between the two indicates how strongly clicks from each time point influenced the final decision. Thick solid lines were obtained from the rat's responses; the thickness of the line represents the standard error. Narrow shaded lines were predicted by the best-fit model. (B) Accurate estimates of  $\sigma_s^2$  and  $\sigma_i^2$ : actual performance (fraction correct) on short-duration trials in which one side had one pulse and the other side had two pulses (i.e., trials for which performance was dominated by  $\sigma_s^2$  and  $\sigma_i^2$ ), versus performance predicted by fitting the model to trials with multiple pulses on each of the two sides. Too few trials of the short type to perform this analysis were presented for one of the human visual data sets and all human auditory data sets. Solid circles are individual rats, and open diamonds are individual humans. The red cross shows the mean and standard error across subjects. The accurate estimates of  $\sigma_s^2$  and  $\sigma_i^2$  suggested by the good predictions also suggest that  $\sigma_a^2$  was estimated accurately, because the sum  $\sigma_s^2 + \sigma_i^2 + \sigma_a^2$  is tightly constrained by the data (fig. S12). (C) Assessing  $\sigma_a^2$ ,  $\phi$ , and  $\tau_\phi$ : Trials were divided into groups, with sensory evidence and sensory noise controlled by keeping the total number of right clicks and the total number of left clicks fixed within each group. Performance within each group was then dominated by  $\sigma_a^2$ ,  $\phi$ , and  $\tau_\phi$ . (C) shows the performance of an example rat, averaged across trial groups and relative to the overall mean of each group, as a function of stimulus duration. The red line is the prediction from the best-fit model, with  $\sigma_a^2 = 0$ .

uses the full information about each trial's richly detailed stimulus (22), is a powerful approach for precisely quantifying multiple properties of decision-making processes. The approach provided strong evidence that rats can indeed gradually accumulate evidence for decision-making (23–26), thus establishing that this important cognitive phenomenon can be studied in a widely available animal model that is amenable to a rapidly growing arsenal of molecular tools.

With its capacity to provide moment-by-moment estimates of the temporal evolution of the accumulator, the approach will combine particularly well with neurobiological measurements. The model used for analysis can be readily expanded to consider and quantify further decision-making parameters, and the approach is easily generalized to different species, sensory modalities, and types of decision-making, including value-based decision-making (8, 27).

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## Supplementary Materials

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Modeling Methods

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A close-up portrait of Deborah S. Jin, a woman with long dark hair and bangs, resting her chin on her hand. She is wearing a dark blue sweater. The background is a plain, light grey.

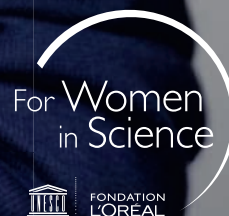
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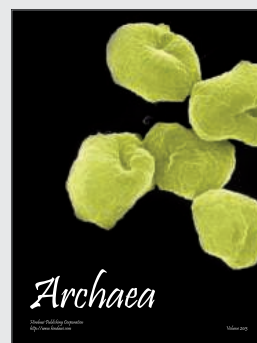
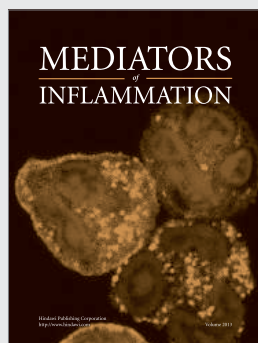
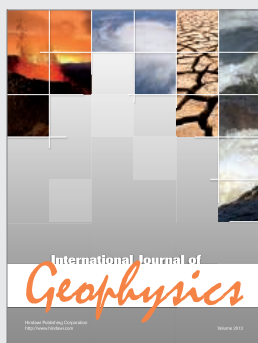
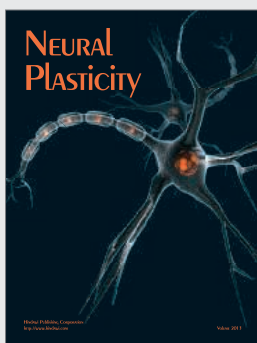
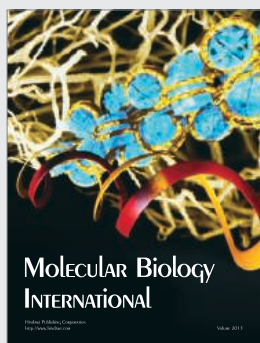
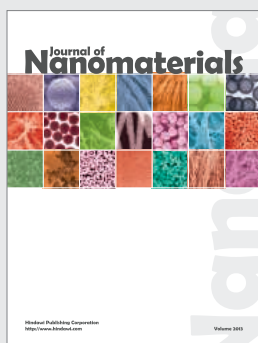
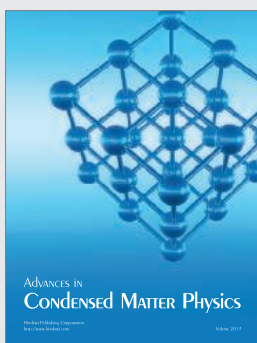
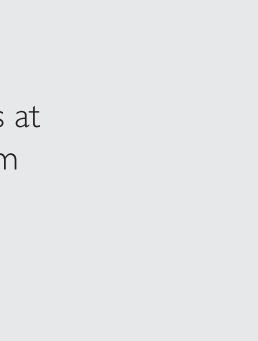
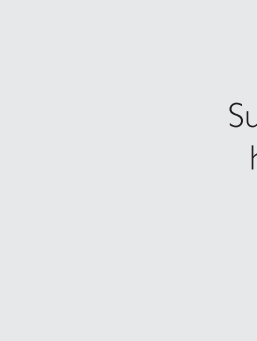
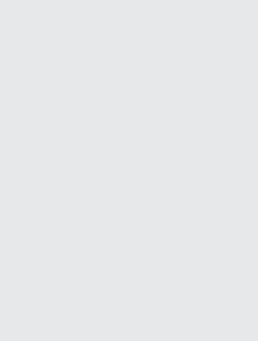
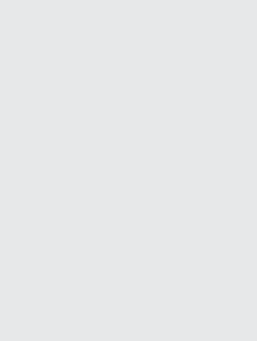
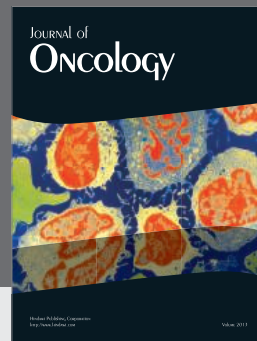
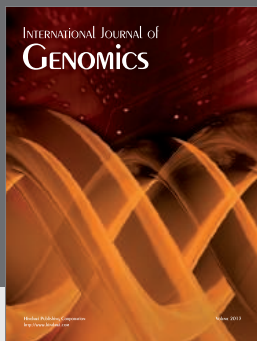
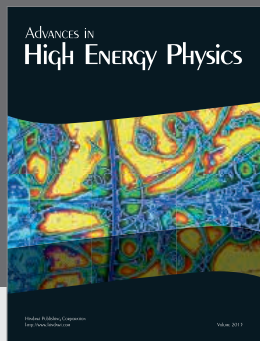
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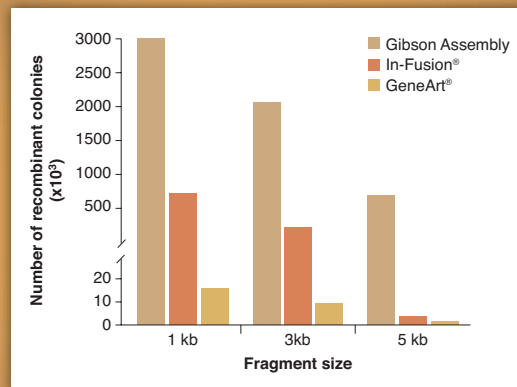
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From the journal *Science*



ScienceCareers.org

UNIVERSITY OF  
**Nebraska**  
Lincoln

The Institute of Agriculture and Natural Resources, Department of Agronomy and Horticulture is pleased to accept applications for a **Plant Molecular Physiologist**, academic year (9-month), tenured or tenure-leading position at the Assistant, Associate, or Full Professor rank with 20% teaching and 80% research responsibilities. Rank and tenure will be based on experience and credentials of the successful applicant. The position will have a joint appointment in the Center for Plant Science Innovation. This incumbent will lead a discovery research program focused on elucidating the physiological, genetic, and metabolic mechanisms involved in the plant's response to environmental stress. Research focus will be on the identification of metabolic processes and networks critical for mitigating environmental stress at the organismal and cellular level. Collaboration with faculty in the Stress Biology Initiative to introduce and evaluate candidate genetic traits for efficacy and commercial potential is expected.

Research responsibilities include using model organisms and/or crop species to provide a path from discovery research to application, and provide the basis for the development of crops through breeding and/or biotechnology with improved water and nitrogen use efficiency, and salt tolerance. Teaching responsibilities include contributing to undergraduate and graduate teaching programs and curriculum development in the Plant Sciences. Additional responsibilities include maintaining a vigorous research program supported through external grants and contracts; publishing research results in refereed scientific journals and presenting results at professional meetings; and advising undergraduate and graduate students.

Requires a Ph.D. or Ph.D. degree in place by date of employment in plant science, botany, biochemistry, agronomy, horticulture, or related field.

To view complete position details and apply for this position, go to the UNL Employment website: <http://employment.unl.edu>. Search for requisition number **F\_130108**. Click on "Apply to this Job," complete the form and then attach a letter of application, an overview of research and teaching experience and interests, and curriculum vitae. Arrange for 3 letters of reference to be sent to: [cwendt1@unl.edu](mailto:cwendt1@unl.edu). Review of applications will begin on **June 7, 2013** and continue until the position is filled or the search is closed.

*The University of Nebraska has an active National Science Foundation ADVANCE gender equity program, and is committed to a pluralistic campus community through affirmative action, equal opportunity, work-life balance, and dual careers.*

## SH SERRA HÜNTER PROGRAMME

The Serra Hünter Programme<sup>3</sup> announces an opening for 75 senior positions in the Catalan public universities in the following research fields: **PHYSICS 4, CHEMISTRY 6, MATHEMATICS 2, BIOLOGY 8, MEDICINE 6, LAW 2, SOCIAL SCIENCES 6, PHILOLOGY 7, HUMANITIES 6, ARTS 2, ARCHITECTURE 3, ENGINEERING 12, PEDAGOGY 7, PSYCHOLOGY 2, OTHER 1.**

Minimum requirements are a PhD degree, and a contrastable academic background. Only those applications with excellent research records, leadership capabilities and, preferentially, with international exposure at the doctoral or post-doctoral level, will be considered.

Successful applicants, unless otherwise stated, will have a permanent contract with one of the Catalan universities, and are expected to cooperate with the existing research groups and develop new research lines, complementary to those already in place. Salaries will be in line with those paid by the Catalan universities, plus (subject to negotiation)

an additional amount for those candidates with outstanding research performance, or a start-up grant, if appropriate. The successful applicants will be evaluated after a three year period, and subsequently every six years. A positive evaluation may lead to a consolidation of the additional payments.

### Applications and deadline

Applications must be submitted electronically via the Serra Hünter Programme website. The website provides all the information necessary for application. The deadline is April 30, 2013.

→ For further details please visit [serrahunter.gencat.cat](http://serrahunter.gencat.cat)

3. The Serra Hünter Programme is funded by the Government of Catalonia and by the seven public universities of Catalonia: University of Barcelona (UB), Universitat Autònoma de Barcelona (UAB), Universitat Politècnica de Catalunya - BarcelonaTech (UPC), Pompeu Fabra University (UPF), University of Lleida (UdL), University of Girona (UdG), and Rovira i Virgili University (URV), its main aim being to hire high-quality staff for the Catalan universities.



## FACULTY POSITIONS AVAILABLE

The Virginia Bioinformatics Institute performs world-class informatics research in life sciences, social sciences, and human health by integrating theory, modeling and simulation with computational and experimental science in a transdisciplinary, team science research environment. The Institute's mission is to solve some of society's most important problems affecting life sciences, social sciences and human health through transdisciplinary informatics research and education.

**ABOUT VBI.** Established in 2000 by the Commonwealth of Virginia, the Institute is a part of Virginia Tech and is located on its Blacksburg, Virginia campus. It has a 154,000 sq. ft. research facility with state-of-the-art core laboratory and high performance computing facilities as well as research offices in the National Capital Region, in Ballston, VA. VBI has developed distinctive informatics research capabilities and has achieved a solid foundation for further growth. The Institute is pursuing its vision to become a world leader in transdisciplinary informatics research which will involve developing an increased capacity for the analysis of interacting complex systems. In practice, this means making transformative discoveries, solving important problems, and developing the next generation of transdisciplinary researchers. The Institute influences public policy and transitions scientific research into use. ([www.vbi.vt.edu](http://www.vbi.vt.edu))

**ABOUT TEAM SCIENCE.** VBI strongly emphasizes team science and organizes research outside of boundaries of academic disciplines. Research programs represented at VBI assemble to meet the specific needs of those programs; it is a flexible environment that rewards the notion of a problem-solving capability on the move. Extensive national and international collaborations complement the expertise of the research groups, including significant interactions with academic medical research centers.

VBI seeks to complement its existing programs in two main areas:

**ADVANCED COMPUTING AND INFORMATION LABORATORIES.** ACIL is focused on advancing informatics methods in massively interacting systems. The programmatic theme connecting the topical foci is employment of high-performance, pervasive and data-intensive approaches to complex biological and socially coupled systems. Affiliated laboratories of ACIL include the Nutritional Immunology and Molecular Medicine Laboratory (NIMML), Network Dynamics and Simulation Science Laboratory (NDSSL) and the Social and Decision Informatics Laboratory (SDIL). Domain research topics range over, and often integrate, systems biology to detailed regional population behavior and economic modeling. In addition, ACIL labs engage in related basic research in mathematics, theoretical computer science, network science, and network-centric high-performance computing systems.

**CYBERINFRASTRUCTURE.** The CI research program is an integrative research team that develops, evolves, deploys and uses large-scale information systems applying the principles of cyberinfrastructure to aggregate and curate data, computational infrastructure, and people in support of health sciences research. Through its research and development activities, CI currently is responsible for many public resources for curated, diverse biological, genome-scale data from various infectious disease systems, and implemented the processes, systems, interfaces and databases in support of such activities, as well as conducting cutting-edge research in the areas of new discoveries.

**ABOUT THE POSITIONS.** To support the growth of its transdisciplinary research portfolio, VBI is seeking team oriented, visionary researchers, to provide leadership and strategic focus. The successful candidates will hold the rank of associate or full professor based solidly on exceptional research accomplishments including an active, extramurally-funded research program that will augment VBI's current strengths. The successful candidates will be fully engaged in leading transdisciplinary research aimed at solving complex informatics based problems in the life sciences, social sciences and human health.

Successful candidates will be placed in either the ACIL or CI research programs noted above based on research interests, credentials and programmatic need. Accomplished faculty with either computational or experiential laboratory research programs are encouraged to apply. A competitive allowance for laboratory start up and operational support will be provided. The transfer of intact research teams is encouraged.

Based on the successful candidate's experience and credentials, tenured appointments with relevant academic departments at Virginia Tech will be considered; such appointments require approval by the relevant department faculty committees and university officials. Alternatively, appointments may be made as non-tenure track Special Research Faculty, with the rank appropriate to the individual's qualifications and experiences, such as associate or full research professor. For more information on the University faculty ranks, please consult the Faculty Handbook located at [http://www.provost.vt.edu/faculty\\_handbook/faculty\\_handbook.html](http://www.provost.vt.edu/faculty_handbook/faculty_handbook.html).

### QUALIFICATIONS.

- Ph.D. in life sciences, microbiology, genetics, computer science, engineering, biotechnology, mathematics, statistics or related appropriate field(s), with a biological, social science or human health focus.
- Demonstrated interest or experience at the interface of the informatics or computational aspects of the life sciences.
- Reputation as an accomplished leader in the development of important research programs complementing VBI's research mission.
- Experience leading and mentoring in a diverse, team science environment as a project investigator or co-project investigator.

### PREFERENCES.

- Evidence of exceptional performance on extramurally funded collaborative research efforts, preferably in life sciences, social sciences, or human health with a shared informatics perspective.
- Experience with high-performance computing, high-volume data generation, visualization and analysis.

### TO APPLY.

Visit [www.jobs.vt.edu](http://www.jobs.vt.edu) and see SR0130027 OR Contact: Lynn Byrd, Director of Talent Management [byrd@vbi.vt.edu](mailto:byrd@vbi.vt.edu) or 540-231-1904  
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## Position Available: Director, NASA Astrobiology Institute

NASA seeks a new Director for the NASA Astrobiology Institute (NAI). The ideal candidate will be an internationally recognized scientist with proven experience in leading large, multi-disciplinary, multi-site research programs or projects, possessed with a vision for leading the Institute into the future. Established in 1998 as part of NASA's Astrobiology Program, the NAI is a collaboration between NASA, US academic institutions, and foreign institutions, governments and research organizations – and is composed of over 800 US scientists and hundreds of researchers abroad. The NAI, currently headquartered at NASA Ames Research Center in the heart of California's Silicon Valley, functions as a virtual institute, its members linked by modern information technologies.

The NAI Director, a member of the federal Senior Executive Service (SES), is both the senior scientific officer and chief operating officer of the NAI. U.S. citizenship is required. Interested applicants should apply directly to USAJobs to vacancy number **AR13S0001** at <http://www.usajobs.gov/GetJob/ViewDetails/339239100>. This website also provides additional details and the application deadline.

*NASA Ames Research Center does not discriminate in employment on the basis of race, color, religion, sex, national origin, political affiliation, sexual orientation, gender identity, marital status, disability and genetic information, age, membership in an employee organization, or other non-merit factor.*



## Call for applications for FY2014 Foreign Postdoctoral Researcher (FPR) RIKEN, Japan



RIKEN is one of Japan's largest research organizations with institutes and centers in various locations. RIKEN carries out advanced basic and applied research in a wide range of fields, including physics, chemistry, medical science, biology, and engineering.

RIKEN is now accepting applications for the position of Foreign Postdoctoral Researcher (FPR) for FY2014. This position is for foreign scientists who have demonstrated creative and innovative ideas and who can be expected to achieve broad international recognition in the future.

The FPR program will provide an opportunity for foreign scientists to apply their creative and innovative ideas, under the direction of RIKEN's laboratory heads, to research currently being conducted at RIKEN.

Applicants must have a PhD in the natural sciences awarded in or after 2008.

Salary is 487,000 yen per month. An annual research budget of 1 million yen is allocated to the host laboratory.

**Application Deadline: 5pm on Friday, May 31, 2013 (Japan Standard Time)**

For details, see <http://www.riken.jp/careers/programs/fpr/>

FPR Desk

Global Relations Office, RIKEN

2-1 Hirosawa, Wako, Saitama 351-0198 Japan

Fax: +81-48-463-3687

Email: [fpr@riken.jp](mailto:fpr@riken.jp)

THE UNIVERSITY OF HONG KONG



Founded in 1911, The University of Hong Kong is committed to the highest international standards of excellence in teaching and research, and has been at the international forefront of academic scholarship for many years. The University has a comprehensive range of study programmes and research disciplines spread across 10 faculties and about 100 sub-divisions of studies and learning. There are over 23,400 undergraduate and postgraduate students coming from 50 countries, and more than 1,800 members of academic and academic-related staff.

### Tenure-Track Assistant Professor in the Department of Physics (Ref.: 201300154)

Applications are invited for a tenure-track appointment as Assistant Professor in the Department of Physics, from as soon as possible. The position will initially be made on a three-year term basis, with the possibility of renewal subject to mutual agreement.

The position will be in the field of Experimental Particle/Nuclear Physics. Existing research activities in the Department include astrophysics, condensed matter physics, neutrino physics, and materials science. Candidates with excellent qualifications and commitment to high-quality research and excellence in teaching are encouraged to apply. The appointee is expected to set up his/her research laboratory.

A globally competitive remuneration package commensurate with the appointee's qualifications and experience will be offered. At current rates, salaries tax does not exceed 15% of gross income. The appointment will attract a contract-end gratuity and University contribution to a retirement benefits scheme, totalling up to 15% of basic salary, as well as leave, and medical benefits. Housing benefits will be provided as applicable.

For enquiries about the existing research activities and the specific job requirements, please write to Professor K.S. Cheng, Head of the Department of Physics (e-mail: [physhead@hku.hk](mailto:physhead@hku.hk)). Interested applicants should submit a completed application form, together with a full C.V., a detailed publication list, a research plan, and a statement on teaching philosophy to [scphy@hku.hk](mailto:scphy@hku.hk). Please indicate clearly "Ref.: 201300154 (Tenure-Track Assistant Professor in Department of Physics)" in the subject of the e-mail. Application forms (341/1111) can be obtained at <http://www.hku.hk/apptunit/form-ext.doc>. Further particulars can be obtained at <http://jobs.hku.hk/>. **Closes June 30, 2013.**

The University thanks applicants for their interest, but advises that only shortlisted applicants will be notified of the application result.

**The University is an equal opportunity employer and is committed to a No-Smoking Policy**



University of Connecticut  
Health Center

## Tenure-Track Immunology Faculty Position

The Department of Immunology at the University of Connecticut Health Center seeks an outstanding investigator for a tenure-track position at the Assistant/Associate/Full Professor level. Although all areas of immunology will be considered, we are particularly interested in individuals using molecular, cellular and translational approaches to study immune system function in vivo. Areas of priority include but are not limited to mucosal immunity including the microbiome, innate immunity, signal transduction and transcriptional control, and dendritic cell biology. The new hire will participate in a vibrant Ph.D. training program and have access to a growing translational research community. Salary and start-up funds are highly competitive and outstanding core facilities are available. Applicants must have a Ph.D. or M.D./Ph.D. and for senior appointments a history of sustained extramural funding and a high impact publication record. In addition to the beauty of the picturesque New England countryside, the Hartford area offers a vibrant arts and cultural scene and an exceptional outdoor sports environment.

Applicants should apply at <https://jobs.uchc.edu> search number 2012-067 and submit curriculum vitae, a two-page summary of research interests and the names of three references. Information may also be submitted to Dr. Leo Lefrançois, Ph.D., Chairman, Department of Immunology, UConn Health Center, Farmington, CT, 06030. Email: [immunology@uchc.edu](mailto:immunology@uchc.edu). For further information on UCHC immunology, please visit [immune.uchc.edu](http://immune.uchc.edu).

*UCHC is an Equal Opportunity Employer M/F/V/PwD*



# Programm zur Förderung der Rückkehr des hoch qualifizierten Forschungsnachwuchses aus dem Ausland

Ministerium für Innovation,  
Wissenschaft und Forschung  
des Landes Nordrhein-Westfalen



Sie stehen am Anfang Ihrer Forscherkarriere und möchten mit Ihren herausragenden Ideen zur Bewältigung der großen gesellschaftlichen Herausforderungen auf den Feldern Klima, Energie, Gesundheit oder Ernährung beitragen? Der Forschungsstandort Nordrhein-Westfalen bietet Ihnen die Chance zum Aufbau und zur Leitung einer selbstständigen Nachwuchsgruppe an einer Hochschule Ihrer Wahl in Nordrhein-Westfalen.

Im Falle einer erfolgreichen Bewerbung sind dafür über einen Zeitraum von fünf Jahren bis zu 1,25 Mio. EUR vorgesehen. Die Leitungsposition ist mit Entgeltgruppe 15 TV-L – vergleichbar W2 – dotiert. Sie erhalten eine personengebundene Finanzierungszusage und etablieren Ihre Nachwuchsgruppe an einer Hochschule Ihrer Wahl in Nordrhein-Westfalen, welche Ihnen die beste Zukunftsperspektive und eventuell auch Tenure-Track bietet.

Der Beginn dieser Förderung ist für 2014 vorgesehen und steht unter dem Vorbehalt der Freigabe durch den Haushaltsgesetzgeber des Landes Nordrhein-Westfalen.

Sie forschen derzeit außerhalb Deutschlands und verfügen über eine Promotion, die zwischen zwei bis sechs Jahre zurückliegt (bei Medizinerinnen und Medizinern zwei bis neun Jahre). Ihr Lebensmittelpunkt lag vor dem Auslandsaufenthalt in Deutschland, und Sie können insgesamt mindestens 24 Monate erfolgreicher wissenschaftlicher Forschung außerhalb Deutschlands vorweisen. Wenn dies alles auf Sie zutrifft, freuen wir uns auf Ihre Bewerbung unter

[www.rueckkehrerprogramm.nrw.de](http://www.rueckkehrerprogramm.nrw.de)

Nähere Informationen zu Bewerbungsunterlagen sowie eine detaillierte Beschreibung des Programms finden Sie auf der angegebenen Internetseite.

Bitte reichen Sie Ihre Bewerbungsunterlagen bis zum **31. Mai 2013 (Deadline)** online ein.

Das Land Nordrhein-Westfalen fördert die berufliche Entwicklung von Frauen. Bewerbungen von Frauen werden daher besonders begrüßt. Bewerbungen geeigneter schwerbehinderter Menschen sind erwünscht.

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[www.wissenschaft.nrw.de](http://www.wissenschaft.nrw.de)

RUPRECHT-KARLS-  
UNIVERSITÄT  
HEIDELBERG



The Faculty of Chemistry and Earth Sciences of the Ruprecht-Karls-University Heidelberg invites applications for a

## W3-Professorship of Applied Physical Chemistry (Succession of M. Grunze)

a position to be filled as from the beginning of the winter semester 2013/2014. Candidates are expected to take over teaching and administrative responsibilities in the whole field of Physical Chemistry at the undergraduate and graduate level and should therefore have the appropriate background and experience.

Candidates for the position are expected to have an internationally established record of accomplishments in current research in surface chemistry and surface physics. A participation in the new Centre of Advanced Materials (CAM) would be appreciated. She / he should hold a Habilitation degree or have research credentials at an equivalent level, possibly with relevant industrial experience.

The University of Heidelberg seeks to increase the number of female research and teaching staff and therefore especially encourages qualified women to apply. According to German law, disabled applicants with an equivalent high qualification will be given preference.

Applicants are asked to submit a detailed curriculum vitae, the list of publications, an account of the teaching experience, the relevant documentation of academic degrees as well as a concise summary of current and proposed research activities, along with their five most relevant publications prior the **15.05.2013** to: **Dean of the Faculty of Chemistry and Earth Sciences, Prof. Dr. H. Gebhardt, Im Neuenheimer Feld 234, D-69120 Heidelberg, Germany**

Breakthrough



Careers



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- » Timely Advice and Answers
- » Community, Connections, and More!

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ADVANCING SCIENCE, SERVING SOCIETY

The American Association for the Advancement of Science (AAAS) seeks a **Project Director** to work within the AAAS Dialogue on Science, Ethics, and Religion Program (DoSER). This is a senior position involving supervising DoSER staff and overseeing project activities.

#### Major duties and responsibilities:

- Oversee program activities
- Develop budgets and manage program finances
- Write and edit grant proposals
- Supervise staff and interns
- Develop new project ideas
- Travel and speak on behalf of program
- Liaise with senior AAAS staff

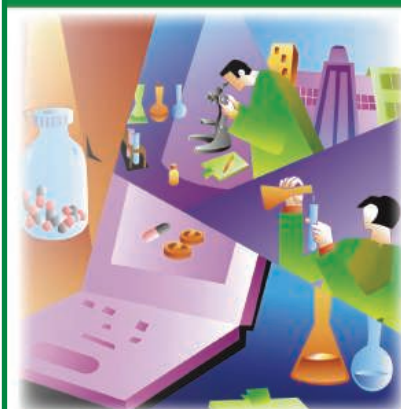
#### Minimum requirements:

- Extensive university or college level training leading to a PhD degree in a scientific field accompanied by subsequent professional experience
- Financial management experience
- Interest in and familiarity with issues at the intersection of science and religion
- Public Speaking experience
- Experience organizing public events
- Strong technology skills in contemporary software packages, particularly Microsoft Office
- Coursework in theology or religious studies preferred
- Ability to travel at least four times annually

Please visit our career resources website, <http://www.aaas.org/careercenter/employmentataaas/> to get more information about careers at AAAS and to apply to AAAS online.

*AAAS is an Equal Opportunity Employer.*

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## Canada Excellence Research Chair in Quantum Materials and Devices

The **University of British Columbia** has been awarded a Canada Excellence Research Chair (CERC) in Quantum Materials and Devices. This major program is designed to attract a researcher of the highest calibre, with \$10 million in CERC research support over seven years, plus over \$30 million in new investments by the University.

UBC has a strong contingent of Condensed Matter researchers in the Department of Physics and Astronomy, an interdisciplinary research environment in the Advanced Materials and Process Engineering Laboratory (AMPEL), and is supporting this field through the development of a Quantum Matter Institute, now partnered with the Max Planck Society in Germany through major bilateral investments in collaborative research. Coupled to this CERC initiative, UBC is committed to an additional three faculty positions in complementary fields, additional research funding, and major expansion of research space.

Applicants must have a PhD Degree or equivalent, in Physics or in related areas of Chemistry, Materials Science or Engineering. An outstanding research record is essential, but could be rooted in industry or national labs as well as academic institutions.

The candidate will be appointed at the level of Professor, but researchers at earlier career stages are welcome to apply, and applicants from research



a place of mind

THE UNIVERSITY OF BRITISH COLUMBIA

backgrounds outside of academic institutions will be considered. The candidate will contribute to the University's commitment to excellence in teaching and research supervision.

UBC hires on the basis of merit and is committed to employment equity. All qualified persons are encouraged to apply. We especially welcome applications from members of visible minority groups, women, Aboriginal persons, persons with disabilities, persons of minority sexual orientations and gender identities, and others with the skills and knowledge to engage productively with diverse communities.

The University is also responsive to the needs of dual career couples and we encourage all qualified candidates to apply. Canada Excellence Research Chairs are open to individuals of any nationality.

Interested individuals are encouraged to contact the Chair of the Search Committee, Simon Peacock, Dean of Science, or our search consultants, Barbara Morrison and Brent Cameron of Odgers Berndtson, at 1-604-685-0261, or [barbara.morrison@odgersberndtson.ca](mailto:barbara.morrison@odgersberndtson.ca) for more information. To apply directly, please email to the above address a copy of your CV, publications list, and a statement of research interests. Review of applications will begin in March/April 2013 and continue until the position has been filled.



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## ASSISTANT/ASSOCIATE PROFESSOR

The Department of Neural and Behavioral Sciences, Pennsylvania State University College of Medicine, is beginning a significant expansion of its faculty. We invite applications from outstanding candidates for multiple tenure-eligible faculty positions at the Assistant or Associate Professor level. The successful candidates will be expected to have, or to establish, an active research program in neuroscience. Exceptional more senior candidates with well-established research programs may also be considered.

In recent years the Department of Neural and Behavioral Sciences has developed excellence in a number of areas including substance abuse and addiction, central control of visceral organs, and vision science. We will consider candidates using innovative approaches to study any aspect of neuroscience, including those whose expertise complements that of our existing programs.

The Department of Neural and Behavioral Sciences provides a competitive start-up package, remodeled laboratory space and excellent core facilities. There are also extensive opportunities for collaborative research and participation in graduate training programs.

Candidates should hold a Ph.D., M.D. or equivalent degree, and are required to send a current curriculum vitae, statement of research interests and goals and information for three references to:

Search Committee c/o Ms. Cathy Hirschbock, Department of Neural and Behavioral Sciences, Penn State University College of Medicine, P.O. Box 850, MC H109, 500 University Drive, Hershey, PA 17033 or email to: [chirschbock@hmc.psu.edu](mailto:chirschbock@hmc.psu.edu)

Materials will be accepted until the positions are filled.

Employment will require successful completion of background check(s) in accordance with University policies.

Penn State is committed to affirmative action, equal opportunity and the diversity of its workforce.



## Young Group Leader Positions in Stem Cell Biology

The Institut Pasteur announces an international call for candidates wishing to establish independent research groups on its Paris, France campus. The recruitments are part of the Revive Laboratory of Excellence (LabEx) programme, recently awarded to the Institut Pasteur on "Stem Cells and Regenerative Biology and Medicine". Candidates will be integrated into the cutting edge interdisciplinary environment provided by the Department of Developmental & Stem Cell Biology. Candidates specializing in the field of stem cells in the context of developmental and cell biology, genetics, epigenetics, regeneration, translational research and ageing are encouraged to apply.

To be eligible, candidates must have defended their PhD on or after May 31, 2005 (women with children are eligible up to 10 yrs after their Ph.D). Successful candidates will be appointed as head of a group of up to 6 people for a period of 5 years. The budget (up to 1,500,000€ over 5 years) includes the salary for the group leader, a three-year postdoctoral position, a technician's position, part-time secretarial assistance, a substantial contribution to running costs and equipment, and access to on-campus facilities including state-of-the-art technology core facilities. Candidates should send their formal applications by E-mail to the Director of Scientific Evaluation, Prof. Alain Israël, at the Institut Pasteur ([g5revive@pasteur.fr](mailto:g5revive@pasteur.fr)). The deadline for applications is **May 31st, 2013**. Short-listed candidates will be contacted for interview to be scheduled for beginning of September 2013 and recruitment decisions announced by October 2013. Further information on the Revive program can be found at <http://www.pasteur.fr/revive>

Applicants should provide the following (in order) in **a single pdf file**: (1) A brief introductory letter of motivation, including the name of the proposed group. Candidates are encouraged to contact the coordinator of the Revive programme Shahrugim Tajbakhsh ([shaht@pasteur.fr](mailto:shaht@pasteur.fr)). (2) A Curriculum Vitae and a full publication list. (3) A description of past and present research activities (up to 5 pages with 1.5 spacing; Times 11 or Arial 10 font size). (4) The proposed research project (up to 10 pages with 1.5 spacing; Times 11 or Arial 10 font size). (5) The names of 3 scientists from whom letters of recommendation can be sought, together with the names of scientists with a potential conflict of interest from whom evaluations should not be requested.



## POSITIONS OPEN

### UNIVERSITY OF MAINE

College of Natural Sciences, Forestry, and Agriculture  
Department of Molecular and Biomedical Sciences

The Department of Molecular & Biomedical Sciences (MBMS) seeks a 50% research/50% teaching, tenure-track, academic year, **ASSISTANT PROFESSOR** of Microbiology, effective September 2013. The successful applicant will teach and provide research opportunities for undergraduate students in capstone research and independent study and for graduate students. The successful candidate will develop and maintain an externally funded research program as well as teach undergraduate Microbiology, another specialty in Microbiology, and develop a graduate course in their specialty. The specific research emphasis of this position is flexible and may include, but is not limited to, the use of genomic and proteomic approaches to study the host-pathogen interactions, response to pathogenic infection, or microbial pathogenesis, quorum sensing and other factors in microbial population dynamics. The University has a state-of-the-art zebrafish facility and is building a core of investigators applying genomics approaches to biomedical research. A Ph.D. in Microbiology, or a relevant field, is required by date of hire. Postdoctoral research experience is also required.

To apply: Please send cover letter, curriculum vitae, research plans, a statement of teaching philosophy, and names and addresses of three references to:

**Dr. Carol Kim (e-mail: carolkim@maine.edu), Microbiologist Search Committee, Department of Molecular & Biomedical Sciences, 5735 Hitchner Hall, University of Maine, Orono, ME 04469-5735.**

Further information may be found at [website: http://www.umaine.edu/biomed](http://www.umaine.edu/biomed). Review of applications will begin April 5, 2013 and continue until the position is filled. Incomplete applications cannot be considered. Appropriate background checks will be required.

On January 1, 2011, University of Maine (UMaine) became a tobacco-free campus. Information regarding UMaine's tobacco-free policy is online at [website: http://umaine.edu/tobaccofree/](http://umaine.edu/tobaccofree/).

*The University of Maine, an Equal Employment Opportunity/Affirmative Action Employer, seeks to employ outstanding people who contribute to the rich cultural diversity expected in a university setting. All qualified individuals are encouraged to apply.*

## TENURE-TRACK FACULTY POSITIONS IN CANCER

**The Cancer Center at Weill Cornell Medical College and NewYork-Presbyterian Hospital  
New York, New York 10065**

The Cancer Center, under the direction of **Lewis C. Cantley, Ph.D.**, and in collaboration with the relevant basic science and academic departments of Weill Cornell Medical College seeks applications from outstanding investigators and academic physicians with basic science and translational research interests.

Appointments are available at several academic levels, ranging from tenure-track **ASSISTANT PROFESSOR** to **ASSOCIATE/FULL PROFESSOR**. Interests or an active program in cancer research that integrates with the Center's focus on precision medicine is also an important criterion for faculty candidates.

Qualified candidates will have an advanced degree, and candidates for senior level positions will have a strong record of independent scientific accomplishments, and a solid extramural funding base.

The Cancer Center offers state-of-the-art facilities and shared resources in the newly constructed Belfer Research Building that is scheduled to open in early 2014. Senior applicants will also have opportunities for leadership positions within the Cancer Center, the organizational unit at Weill Cornell that coordinates basic, clinical, and population cancer research.

Interested applicants should forward curriculum vitae, statement of interests, and the names and contact information for three references to **Jane Lyons, e-mail: jal2030@med.cornell.edu**, Office of the Cancer Center Director.

*Weill Cornell Medical College is an Affirmative Action/Equal Opportunity Employer.*

## POSITIONS OPEN

University of Hawaii at Manoa Cancer Center: **ASSISTANT RESEARCHER** (Cancer Epidemiology Program), **Position #70038**, Tenure-track, Continuous recruitment. Refer to [website: http://www.pers.hawaii.edu/wuh/](http://www.pers.hawaii.edu/wuh/) for complete information. *The University of Hawaii is an Equal Opportunity/Affirmative Action Institution. Women and minorities are encouraged to apply.*

University of Hawaii at Manoa Cancer Center: **FULL RESEARCHER** (Cancer Epidemiology Program), **Position #88357**, Tenure-track, Continuous recruitment. Refer to [website: http://www.pers.hawaii.edu/wuh/](http://www.pers.hawaii.edu/wuh/) for complete information. *The University of Hawaii is an Equal Opportunity/Affirmative Action Institution. Women and minorities are encouraged to apply.*

## POSITION ANNOUNCEMENT

The College of Veterinary Medicine at Mississippi State University (MSU) is seeking applications for a tenure-track **ASSISTANT PROFESSOR** position in the Department of Basic Sciences. The successful applicant must have research experience in type 2 diabetes and/or related cardiovascular conditions and an interest in research on human health disparities involving these health issues. Health disparities research has been identified as a university-wide research priority. The Center for Environmental Health Sciences in the College of Veterinary Medicine is active in health disparities research, and the role of exposure to environmental chemicals (pesticides, for example) in the occurrence of health disparities is a unifying theme within the Center. The successful applicant will have at least one active transferable research grant and a record of publishing consistently in high-quality journals. The successful applicant will be expected to participate in our instructional programs for graduate (M.S. and Ph.D.) and/or D.V.M. students. Minimum qualifications include a Ph.D. with postdoctoral research experience as well as training and publications in type 2 diabetes and/or cardiovascular disease. Salary will be commensurate with experience. Applications must be received by April 22, 2013 to be considered. To apply, please send curriculum vitae, names and contact information for three persons who can provide letters of reference, and a brief (one page or less) statement of research plans and goals, which includes identification of the current research funding as well as teaching philosophy to: **Dr. Matthew Ross, Chair of Search Committee, Department of Basic Sciences, College of Veterinary Medicine, P.O. Box 6100, Mississippi State, MS 39762.** E-mail communication is preferred (e-mail: [mross@cvm.msstate.edu](mailto:mross@cvm.msstate.edu)). Applicants must also complete the online application process at the MSU Human Resources Management [website: https://www.jobs.msstate.edu/applicants/jsp/shared/frameset/frameset.jsp?time=1357508611696](https://www.jobs.msstate.edu/applicants/jsp/shared/frameset/frameset.jsp?time=1357508611696). The position is listed as Assistant Professor and the department is listed as CVM Basic Science Department. *MSU is an Affirmative Action/Equal Opportunity Employer, and members of minority groups who are underrepresented in the sciences are particularly encouraged to apply.*

## POSTDOCTORAL/RESEARCH ASSOCIATE

A Postdoctoral/Research Associate position is available to participate in NIH-funded project to study the molecular mechanisms of lung injury induced by hyperoxia, in relation to human lung diseases such as ALI/ARDS. The successful candidate will have a Ph.D. in the areas of Molecular Toxicology and/or Lung Biology. She or he will use transgenic and knockout mice, cell lines, and apply state-of-the-art molecular and cell biology techniques such as cloning, molecular analyses of genetic variants, site-directed mutagenesis, next generation sequencing, RNA-Seq, ChIP-Seq, microarray, 32P-postlabeling, electrophoretic mobility shift assays, plasmid isolation, real-time RT-PCR, chromatin immunoprecipitation, transfections, Western blotting, microarrays, etc. Please send curriculum vitae to: **Dr. Bhagavatula Moorthy, Department of Pediatrics, Baylor College of Medicine, Houston, TX (e-mail: [bmoorthy@bcm.edu](mailto:bmoorthy@bcm.edu)).**



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